Appendices

Appendix A
Correspondence and Documentation

Environmental Protection Agency Authorization Letter for Section 18 Emergency Exemption under the Federal Insecticide, Fungicide, Rodenticide Act

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

Mr. James R. Hess
Associate Director, Operations
U.S. Department of the Interior
Bureau of Reclamation

Date Issued: August 26, 2010
Interim Report(s) Due: August 26, 2011, August 26, 2012
Final Report Due: August 26, 2013
Expiration Date: August 26, 2013
File Symbol: 09-DI-01

Attn: James Hess

The Environmental Protection Agency hereby grants a quarantine exemption under the provisions of section 18 of the Federal Insecticide, Fungicide, and Rodenticide Act, as amended, to the U.S. Department of the Interior, Bureau of Reclamation for the use of the unregistered active ingredient *Pseudomonas fluorescens*, formulated as MOI-401-EP and/or MOI-401-SDP (aka Zequanox) in three lower Colorado River Dams (Hoover, Davis, and Parker, and their associated power generation facilities) and piped-irrigation water distribution systems of the Lower Colorado River, to control invasive Zebra Mussels (*Dreissena polymorpha*) and Quagga Mussels (*Dreissena bugensis*). This quarantine exemption is subject to the conditions set forth in your request, as well as the following conditions and restrictions:

1. The U.S. Department of the Interior, Bureau of Reclamation is responsible for ensuring that all provisions of this quarantine exemption are met. It is also responsible for providing interim and final reports about the results and effectiveness of this exemption and information about any adverse events resulting from it. This information must be submitted to EPA headquarters and the EPA Region 9 office.

2. The unregistered products MOI-401-EP or MOI-401-SDP (aka Zequanox), manufactured by Marrone Bio Innovations, Inc., may be applied. The terms of this authorizing letter as well as those contained in the use directions submitted by Marrone Bio Innovations, Inc. (registrant) on 8/24/10 must be used.

3. The pesticide product may be applied under the rehabilitation and/or settlement maintenance treatment scenarios using standard aquatic pesticide application equipment or similar equipment commonly used for chemical injection in drinking water treatment. The pesticide product will be contained and transported in totes or appropriate plastic chemical application barrels.

Environmental Protection Agency Authorization Letter for Section 18 Emergency Exemption under the Federal Insecticide, Fungicide, Rodenticide Act

4. Regardless of the treatment scenario employed, the concentration of the active ingredient will not exceed 200 mg/L, continuously applied for no longer than 24 hours - not to exceed a combined (non-contiguous) total of 24 hours per 4-week period.

5. All personnel who work with MOI-401-EP and MOI-401-SDP must be certified pesticide applicators and must use and be trained in the use of required and appropriate personal protective equipment, as per OSHA standards, the Material Safety Data Sheets (MSDS), and the product label. Working with MOI-401-EP and MOI-401-SDP (including handling and storage, process replenishment and housekeeping activities) requires worker to use appropriate eye and face protection, gloves, and impervious clothing.

6. Prior to commencing treatment process work on-site, the Bureau of Reclamation will prepare a job hazard analysis, which will detail emergency response and spill response measures, to include emergency phone numbers and locations of nearby emergency facilities. In addition, pre-job safety briefings will be conducted prior to each treatment.

7. An exemption from tolerance will be established for this strain of *Pseudomonas fluoresecens*.

8. The proposed use is not expected to pose a significant risk to the environment, to ground and surface water, or to non-target organisms.

9. Any unused unregistered product must either be returned to the manufacturer or distributor (unopened containers) or disposed of in accordance with Resource Conservation and Recovery Act regulations.

10. Interim and final reports should indicate information on the actual product used and the effectiveness of the treatment(s) relative to life stages treated and performance in differing aquatic environments (i.e. flowing or static).

Any future correspondence in connection with this exemption should refer to file symbol 09-DI-01.

Steven Bradbury, Director
Office of Pesticide Programs

Date: 8/26/10

cc: EPA Region 9
Patti TenBrook

Appendix B

Federal Insecticide, Fungicide, Rodenticide Act
Section 18 Emergency Exemption Project File Docket

United States Department of the Interior

BUREAU OF RECLAMATION
Washington, D.C. 20240

JAN 27 2010

IN REPLY REFER TO:

86-69000
PRJ-1.10

EXPRESS MAIL ONLY

Mr. Tony Britten
Environmental Protection Agency
Office of Pesticide Programs (EMEX)
Room S4900, One Potomac Yard
2777 Crystal Drive
Arlington, VA 22202

Subject: Amendment to Request for Emergency Quarantine Exemption Under Section 18 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for the Molluscicide, MOI 401 (Zequanox™)

Dear Mr. Britten:

On September 4, 2009, the Bureau of Reclamation requested your approval to permit the use of an unregistered pesticide for the purpose of emergency quarantine management of invasive zebra and quagga mussels in accordance with 40 CFR 166.2(b). The exemption will cover use of the *Pseudomonas fluorescens* – based molluscicide, Zequanox™

As a result of subsequent consultations with you and your colleagues, we have amended our original application as follows: (1) reduced the geographic treatment area to the mussel-infested areas of the lower Colorado River, (2) limited treatments to high dilution areas at impacted dams with hydropower plants (i.e., Hoover, Davis and Parker Dams) and piped irrigation systems of the Lower Colorado River where treated water is not returned to natural fish-bearing waters, and (3) identified methods for monitoring the bacterial product in treated waters. These changes are identified in the enclosed amendment.

For further information or any questions you may have, please contact Dr. Curtis Brown, Director, Research and Development, at 303-445-2098, or by e-mail at cbrown@usbr.gov.

Sincerely,

for James R. Hess
Associate Director, Operations

Enclosures – 4

cc: See next page.

cc: Ms. Ann Sibold
Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention
Division (7511P)
1200 Pennsylvania Avenue, N.W.
Washington, DC 20460
(w/encl)

APPENDIX 1

MOI 401 regulatory dossier; contains confidential business information.

Please read instructions on reverse before completing form. Form Approved. OMB No. 2070-0060

Print Form

United States
Environmental Protection Agency
Washington, DC 20460

[X] Registration
[ ] Amendment
[ ] Other

OPP Identifier Number

Application for Pesticide - Section I

1. Company/Product Number	2. EPA Product Manager	3. Proposed Classification
84059-U	Ann Sibold	[X] None [ ] Restricted
4. Company/Product (Name) Marrone Organic Innovations/MOI 401 TGAI	PM#	

5. Name and Address of Applicant *(Include ZIP Code)*

Marrone Organic Innovations, 2121 Second St., Suite B-107
Davis, CA 95618

[ ] *Check if this is a new address*

6. **Expedited Review.** In accordance with FIFRA Section 3(c)(3)(b)(i), my product is similar or identical in composition and labeling to:

EPA Reg. No. _____

Product Name _____

Section - II

[ ] Amendment - Explain below.
[ ] Resubmission in response to Agency letter dated _____
[ ] Notification - Explain below.
[ ] Final printed labels in response to Agency letter dated _____
[ ] "Me Too" Application.
[X] Other - Explain below.

Explanation: Use additional page(s) if necessary. (For section I and Section II.)

MOI 401 Technical Grade Active Ingredient, Pseudomonas fluorescens CL145A (84059-U)

Section - III

1. Material This Product Will Be Packaged In:

Child-Resistant Packaging	Unit Packaging	Water Soluble Packaging	2. Type of Container
[ ] Yes* [X] No	[ ] Yes [X] No	[ ] Yes [X] No	[X] Metal [X] Plastic [ ] Glass [ ] Paper [ ] Other (Specify)
* *Certification must be submitted*	If "Yes" Unit Packaging wgt. / No. per container	If "Yes" Package wgt / No. per container	

3. Location of Net Contents Information	4. Size(s) Retail Container	5. Location of Label Directions
[X] Label [ ] Container	2.5 , 5, 55 gal	[ ] On Label [X] On Labeling accompanying product

6. Manner in Which Label is Affixed to Product: [X] Lithograph [X] Paper glued [ ] Stenciled [ ] Other _____

Section - IV

1. Contact Point *(Complete items directly below for identification of individual to be contacted, if necessary, to process this application.)*

Name	Title	Telephone No. (Include Area Code)
Keith Pitts	VP, Regulatory Affairs	(530) 750-2800

Certification

I certify that the statements I have made on this form and all attachments thereto are true, accurate and complete. I acknowledge that any knowingly false or misleading statement may be punishable by fine or imprisonment or both under applicable law.

6. Date Application Received (Stamped)

2. Signature	3. Title
[signature]	VP, Regulatory Affairs
4. Typed Name: Keith Pitts	5. Date: February 4, 2009

Form Approved OMB Nos. 2070-0060; 2070-0057; 2070-0107; 2070-0122; 2070-0164

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
1200 Pennsylvania Avenue, N.W.
WASHINGTON, D.C. 20460

Certification with Respect to Citation of Data

Applicant's/Registrant's Name, Address, and Telephone Number Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	EPA Registration Number/File Symbol 84059-U
Active Ingredient(s) and/or representative test compound(s) Pseudomonas fluorescens CL145A	Date 2/2/ 2009
General Use Pattern(s) (list all those claimed for this product using 40 CFR Part 158) Microbial Pesticide, Molluscicide, Aquatic	Product Name MOI 401 TGAI

NOTE: If your product is a 100% repackaging of another purchased EPA-registered product labeled for all the same uses on your label, you do not need to submit this form. You must submit the Formulator's Exemption Statement (EPA Form 8570-27).

☐ I am responding to a Data-Call-In Notice, and have included with this form a list of companies sent offers of compensation (the Data Matrix form should be used for this purpose).

SECTION I: METHOD OF DATA SUPPORT (Check one method only)

☐ I am using the cite-all method of support, and have included with this form a list of companies sent offers of compensation (the Data Matrix form should be used for this purpose).

☑ I am using the selective method of support (or cite-all option under the selective method), and have included with this form a completed list of data requirements (the Data Matrix form must be used).

SECTION II: GENERAL OFFER TO PAY

[Required if using the cite-all method or when using the cite-all option under the selective method to satisfy one or more data requirements]

☐ I hereby offer and agree to pay compensation, to other persons, with regard to the approval of this application, to the extent required by FIFRA.

SECTION III: CERTIFICATION

I certify that this application for registration, this form for reregistration, or this Data-Call-In response is supported by all data submitted or cited in the application for registration, the form for reregistration, or the Data-Call-In response. In addition, if the cite-all option or cite-all option under the selective method is indicated in Section I, this application is supported by all data in the Agency's files that (1) concern the properties or effects of this product or an identical or substantially similar product, or one or more of the ingredients in this product; and (2) is a type of data that would be required to be submitted under the data requirements in effect on the date of approval of this application if the application sought the initial registration of a product of identical or similar composition and uses .

I certify that for each exclusive use study cited in support of this registration or reregistration, that I am the original data submitter or that I have obtained the written permission of the original data submitter to cite that study.

I certify that for each study cited in support of this registration or reregistration that is not an exclusive use study, either: (a) I am the original data submitter; (b) I have obtained the permission of the original data submitter to use the study in support of this application; (c) all periods of eligibility for compensation have expired for the study; (d) the study is in the public literature; or (e) I have notified in writing the company that submitted the study and have offered (I) to pay compensation to the extent required by sections 3(c)(1)(F) and/or 3(c)(2)(B) of FIFRA; and (ii) to commence negotiations to determine the amount and terms of compensation, if any, to be paid for the use of the study.

I certify that in all instances where an offer of compensation is required, copies of all offers to pay compensation and evidence of their delivery in accordance with sections 3(c)(1)(F) and/or 3(c)(2)(B) of FIFRA are available and will be submitted to the Agency upon request. Should I fail to produce such evidence to the Agency upon request, I understand that the Agency may initiate action to deny, cancel or suspend the registration of my product in conformity with FIFRA.

I certify that the statements I have made on this form and all attachments to it are true, accurate, and complete. I acknowledge that any knowingly false or misleading statement may be punishable by fine or imprisonment or both under applicable law.

Signature	Date	Typed or Printed Name and Title
	2/2/ 2009	Keith Pitts, Vice President-Regulatory Affairs

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

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DATA MATRIX

Date 2/2/2009	EPA Reg No./File Symbol 84059-U	Page 1 of 2
Applicant's/Registrant's Name & Address Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	Product MOI 401 TGAI	

Ingredient

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
885.1100-1500,830.1800,.6302-7950	M. Koivunen, 12/05/2008, Product Chemistry, Microbial	47640201	Marrone Organic Innovations	OWN	
870.1100	J. Kuhn, 6/03/2008, Acute Oral Toxicity Study	47640202	Marrone Organic Innovations	OWN	
870.1200	J. Kuhn, 6/03/2008, Acute Dermal Toxicity Study	47640203	Marrone Organic Innovations	OWN	
870.1300	L. Carter, 10/14/2008, Acute Inhalation Toxicity Study	47640204	Marrone Organic Innovations	OWN	
870.2400	J. Kuhn, 6/26/2008, Acute Eye Toxicity Study	47640205	Marrone Organic Innovations	OWN	
870.2500	J. Kuhn, 5/07/2008, Acute Dermal Irritation Study	47640206	Marrone Organic Innovations	OWN	
885.3200	K. Monds, 12/23/2008, Acute IV Tox/Path Study	47640207	Marrone Organic Innovations	OWN	
850-2100	C. Fletcher, 7/31/2008, Avian Acute Oral Toxicity Study	47640208	Marrone Organic Innovations	OWN	
885.4200	D. Molloy, 11/26/2008, FW Fish Acute Toxicity Study	47640209	Marrone Organic Innovations	OWN	
885.4240	D. Molloy, 11/26/2008, FW Aquatic Invertebrate Tox Study	47640210	Marrone Organic Innovations	OWN	
885.4240	D. Mayer, 9/19/2008, FW Aquatic Invertebrate Tox Study	47640211	Marrone Organic Innovations	OWN	
885.4200, 885.4240, 885.4280	D. Mayer, 5/21/2008, Misc. Acute Toxicity Studies Summ.	47640212	Marrone Organic Innovations	OWN	
	K. Pitts, 11/19/2008 Endangered Species Assessment	47640213	Marrone Organic Innovations	OWN	
	K.Pitts 11/19/2008, Waiver Request for Microbial Pesticide	47640214	Marrone Organic Innovations	OWN	

Signature	Name and Title	Date
	Keith Pitts, Vice President-Regulatory Affairs	02/02/2009

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

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Applicant's/Registrant's Name & Address Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	Product MOI 401 TGAI	

Ingredient

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			Marrone Organic Innovations	OWN	
			Marrone Organic Innovations	OWN	
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			Marrone Organic Innovations	OWN	

Signature	Name and Title	Date
[signature]	Keith Pitts, Vice President-Regulatory Affairs	02/02/2009

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Print Form

EPA

United States
Environmental Protection Agency
Washington, DC 20460

- [X] **Registration**
- [ ] **Amendment**
- [ ] **Other**

OPP Identifier Number

Application for Pesticide - Section I

1. Company/Product Number	2. EPA Product Manager	3. Proposed Classification
84059-L	Ann Sibold	[X] None [ ] Restricted
4. Company/Product (Name) Marrone Organic Innovations/MOI 401 EP	**PM#**	

5. Name and Address of Applicant *(Include ZIP Code)*

Marrone Organic Innovations, 2121 Second St., Suite B-107
Davis, CA 95618

[ ] *Check if this is a new address*

6. Expedited Review. In accordance with FIFRA Section 3(c)(3)(b)(i), my product is similar or identical in composition and labeling to:

EPA Reg. No. _____

Product Name _____

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- [ ] Amendment - Explain below.
- [ ] Resubmission in response to Agency letter dated _____
- [ ] Notification - Explain below.
- [ ] Final printed labels in response to Agency letter dated _____
- [ ] "Me Too" Application.
- [X] Other - Explain below.

Explanation: Use additional page(s) if necessary. (For section I and Section II.)

MOI 401 End Product, Pseudomonas fluorescens CL145A (84059-L)

Section - III

1. Material This Product Will Be Packaged In:

Child-Resistant Packaging	Unit Packaging	Water Soluble Packaging	2. Type of Container
[ ] Yes* [X] No	[ ] Yes [X] No	[ ] Yes [X] No	[X] Metal [X] Plastic [ ] Glass [ ] Paper [ ] Other (Specify) _____
* *Certification must be submitted*	If "Yes" Unit Packaging wgt. / No. per container	If "Yes" Package wgt / No. per container	

3. Location of Net Contents Information	4. Size(s) Retail Container	5. Location of Label Directions
[X] Label [ ] Container	2.5 , 5, 55 gal	[ ] On Label [X] On Labeling accompanying product

6. Manner in Which Label is Affixed to Product [X] Lithograph [ ] Paper glued [ ] Stenciled [ ] Other _____

Section - IV

1. Contact Point *(Complete items directly below for identification of individual to be contacted, if necessary, to process this application.)*

Name	Title	Telephone No. (Include Area Code)
Keith Pitts	VP, Regulatory Affairs	(530) 750-2800

Certification

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6. Date Application Received (Stamped)

2. Signature	3. Title
	VP, Regulatory Affairs
4. Typed Name Keith Pitts	**5. Date** February 4, 2009

Form Approved OMB Nos. 2070-0060; 2070-0057; 2070-0107; 2070-0122; 2070-0164

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
1200 Pennsylvania Avenue, N.W.
WASHINGTON, D.C. 20460

Certification with Respect to Citation of Data

Applicant's/Registrant's Name, Address, and Telephone Number	EPA Registration Number/File Symbol
Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	84059-L
Active Ingredient(s) and/or representative test compound(s) Pseudomonas fluorescens CL145A	Date 2/2/ 2009
General Use Pattern(s) (list all those claimed for this product using 40 CFR Part 158) Microbial Pesticide, Molluscicide, Aquatic	Product Name MOI 401 EP

NOTE: If your product is a 100% repackaging of another purchased EPA-registered product labeled for all the same uses on your label, you do not need to submit this form. You must submit the Formulator's Exemption Statement (EPA Form 8570-27).

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[Required if using the cite-all method or when using the cite-all option under the selective method to satisfy one or more data requirements]

☐ I hereby offer and agree to pay compensation, to other persons, with regard to the approval of this application, to the extent required by FIFRA.

SECTION III: CERTIFICATION

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I certify that for each exclusive use study cited in support of this registration or reregistration, that I am the original data submitter or that I have obtained the written permission of the original data submitter to cite that study.

I certify that for each study cited in support of this registration or reregistration that is not an exclusive use study, either: (a) I am the original data submitter; (b) I have obtained the permission of the original data submitter to use the study in support of this application; (c) all periods of eligibility for compensation have expired for the study; (d) the study is in the public literature; or (e) I have notified in writing the company that submitted the study and have offered (I) to pay compensation to the extent required by sections 3(c)(1)(F) and/or 3(c)(2)(B) of FIFRA; and (ii) to commence negotiations to determine the amount and terms of compensation, if any, to be paid for the use of the study.

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Signature	Date	Typed or Printed Name and Title
	2/2/ 2009	Keith Pitts, Vice President-Regulatory Affairs

Form Approved OMB No. 2070-0060

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401 M Street, S.W.
WASHINGTON, D.C. 20460

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Date 2/4/2009	EPA Reg No./File Symbol 84059-L	Page 1 of 2
Applicant's/Registrant's Name & Address Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	Product MOI 401 EP	

Ingredient

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
885.1100-1500,830.1800,.6302-7950	M. Koivunen, 12/05/2008, Product Chemistry, Microbial	47640215	Marrone Organic Innovations	OWN	
870.1100	J. Kuhn, 6/03/2008, Acute Oral Toxicity Study	47640202	Marrone Organic Innovations	OWN	
870.1200	J. Kuhn, 6/03/2008, Acute Dermal Toxicity Study	47640203	Marrone Organic Innovations	OWN	
870.1300	L. Carter, 10/14/2008, Acute Inhalation Toxicity Study	47640204	Marrone Organic Innovations	OWN	
870.2400	J. Kuhn, 6/26/2008, Acute Eye Toxicity Study	47640205	Marrone Organic Innovations	OWN	
870.2500	J. Kuhn, 5/07/2008, Acute Dermal Irritation Study	47640206	Marrone Organic Innovations	OWN	
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885.4240	D. Mayer, 9/19/2008, FW Aquatic Invertebrate Tox Study	47640211	Marrone Organic Innovations	OWN	
885.4200, 885.4240, 885.4280	D. Mayer, 5/21/2008, Misc. Acute Toxicity Studies Summ.	47640212	Marrone Organic Innovations	OWN	
	K. Pitts, 11/19/2008 Endangered Species Assessment	NEW STUDY	Marrone Organic Innovations	OWN	NEW STUDY
	K.Pitts 11/19/2008, Waiver Request for Microbial Pesticide	47640216	Marrone Organic Innovations	OWN	

Signature	Name and Title	Date
	Keith Pitts, Vice President-Regulatory Affairs	02/04/2009

Form Approved OMB No. 2070-0060

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401 M Street, S.W.
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DATA MATRIX

Date 2/4/2009	EPA Reg No./File Symbol 84059-L	Page 1 of 2
Applicant's/Registrant's Name & Address Marrone Organic Innovations, 2121 Second Street, Suite B-107, Davis, CA 95618 (530) 750-2800	Product MOI 401 EP	

Ingredient

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			Marrone Organic Innovations	OWN	
			Marrone Organic Innovations	OWN	
			Marrone Organic Innovations	OWN	
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			Marrone Organic Innovations	OWN	
			Marrone Organic Innovations	OWN	

Signature	Name and Title	Date
	Keith Pitts, Vice President-Regulatory Affairs	02/04/2009

APPENDIX 2

OECD consensus document on Information used in the assessment of environmental applications involving pseudomonas.

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Series on Harmonization of Regulatory Oversight in Biotechnology No. 6

CONSENSUS DOCUMENT ON INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING PSEUDOMONAS

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris

49010

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OECD Environmental Health and Safety Publications

Series on Harmonization of Regulatory Oversight in Biotechnology

No. 6

Consensus Document on Information Used in the Assessment of Environmental Applications Involving *Pseudomonas*

Environment Directorate

Organisation for Economic Co-operation and Development

Paris 1997

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FOREWORD

The OECD'S Expert Group on Harmonization of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of ***consensus documents*** which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product.

This document contains information for use during regulatory assessments of environmental applications involving organisms which are fluorescent members of the genus *Pseudomonas* (rRNA group I pseudomonads). The first draft (initially prepared by the United Kingdom) was completed in August 1995. It was then sent to national co-ordinators, nominated by the Expert Group, for technical comments. Following receipt of their comments, and a meeting of a task group established by the Expert Group, it was decided that the document should be revised further. This work was undertaken by Canada, as the lead country.

At its second session, in March 1996, the Expert Group agreed in principle that this document should be recommended for derestriction after the incorporation of certain changes. A revised version was forwarded for consideration to the national co-ordinators who had commented on the previous draft.

The Joint Meeting of the Chemicals Group and Management Committee of the Special Programme on the Control of Chemicals subsequently recommended that this document be made available to the public. It is published on the authority of the Secretary-General of the OECD.

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PREAMBLE

OECD Member countries are moving rapidly towards the commercialisation and marketing of agricultural and industrial products of modern biotechnology. They have therefore identified the need for harmonization of regulatory approaches to the assessment of these products, in order to avoid unnecessary trade barriers.

At the end of 1992, a project on ***Environmental Applications of Modern Biotechnology*** (formerly called ***Industrial Products of Modern Biotechnology***) was initiated under the auspices of the OECD's Environment Policy Committee. The scope of the project includes microorganisms for use in applications such as bioremediation, bioprevention, biomining and bioleaching. Its objective is to assist countries in their regulatory assessment of such applications and to facilitate international harmonization.

The first step in this project was to organize a Workshop, held in Brussels in 1993, to identify the information used by regulatory authorities in OECD countries when assessing these applications. The results, which show considerable commonality among countries, are described in OECD Environment Monograph No. 100, *Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology* (OECD, 1995).

Building on the work of the Brussels Workshop, a second Workshop held in Fribourg, Switzerland, in 1994 identified the ***types*** of information used to address the information elements which had been identified [see Environment Monograph No. 117, *Industrial Products of Modern Biotechnology Intended for Release to the Environment: The Proceedings of the Fribourg Workshop* (OECD, 1996)]. As a result of the Fribourg Workshop, it was shown that much of the information used in regulatory assessments is not case-specific but would be equally applicable to many assessments involving the same or similar host organisms. It was further found that much of this information, such as that related to the biological properties of the host organism, is available in the scientific literature.

In June 1995, at its first session, the Expert Group on the Harmonization of Regulatory Oversight in Biotechnology instituted the development of ***consensus documents*** which are ***mutually acceptable*** among Member countries, as an initial step in efforts to facilitate harmonization. The purpose of these consensus documents is to identify common elements in the safety assessment of environmental applications of modern biotechnology, to encourage information sharing, and to prevent duplication of effort among OECD countries.

The focus of this consensus document is on information which is not case-specific, and which is readily available from the scientific literature, related to fluorescent members of the genus* Pseudomonas *(rRNA group I pseudomonads).

In order to ensure that scientific and technical developments are taken into account, it was agreed that these documents will be updated regularly. Additional areas relevant to the subject of each consensus document will be considered at the time of updating.

Users of this document are invited to provide the OECD with new scientific and technical information, and to make proposals for additional areas to be considered. ***There is a short, pre-addressed questionnaire for this purpose on page 109. The completed questionnaire (or a photocopy) should be returned to the Environmental Health and Safety Division.***

SECTION I – GENERAL INTRODUCTION

This document presents information that is accepted in the scientific literature concerning the known characteristics of fluorescent members of the genus *Pseudomonas* (rRNA group I pseudomonads). Regulatory officials may find this information useful in evaluating and establishing the properties of environmental applications of biotechnology which involve those microorganisms which are the focus of this document. Consequently, a wide range of information is provided without prescribing when the information would or would not be relevant to a specific risk assessment. This document represents a "snapshot" of current information that may potentially be relevant to such assessments. However, Member countries have not yet attempted to put together an exhaustive literature review on all aspects of these organisms.

The genus *Pseudomonas* may potentially be utilised in a number of different engineering applications. These include *in situ* applications such as groundwater reinjection, air sparging, and bioventing. They also include *ex situ* applications such as landfarming, slurry phase remediation, and biopiles. Many of the potential uses under development or envisioned for the genus *Pseudomonas* involve improvement of air, soil or water quality, or cleanup of otherwise intractable environmental contaminants.

In considering information that should be presented on this taxonomic grouping, the Task Group for Environmental Applications of Modern Biotechnology discussed the list of topics developed in the "Blue Book", *Recombinant DNA Safety Considerations* (OECD, 1986), and attempted to pare down that list to eliminate duplications, as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered so they would be easier to understand and use (see Section III).

This effort at refining the exposition of safety considerations in the "Blue Book" for application to the genus *Pseudomonas* has also recognized the importance of a thorough understanding of the characteristics of the particular application for which these organisms will be used. Worker and other human exposures, and environmental exposures, will differ depending on the method of application. This knowledge is likely to affect the types of information on particular taxa that regulatory officials deem relevant in specific risk assessments. Group I pseudomonads are known to display a range of pathogenic and toxicological characteristics in regard to humans, animals and plants. However, even though some of the rRNA group I pseudomonads are known to exhibit pathogenic properties, exposures of and potential impacts on humans, animals and plants may be relatively limited in some circumstances, e.g. when the microorganisms are used in bioreactors of various sorts that have suitable controls on liquid and gaseous emissions, or when other specific mitigation or containment measures are in place. The factors discussed in this document may, therefore, have varying levels of impact on individual risk assessments, depending upon how and where the particular microorganisms are used, i.e. depending on the likely exposures presented by the application.

Given the breadth of information contained in this document, it is hoped that it will be useful not only to regulatory officials as a general guide and reference source, but also to industry and to scientists involved in research.

This document is a consensus document for environmental applications involving fluorescent members of the genus *Pseudomonas* (rRNA group I). Section II is an introduction to the genus *Pseudomonas* and to the species which are the subject of the document. The format of the information is described in Section III, and the information is presented in Section IV. Section V contains the References.

SECTION II – INTRODUCTION TO THE GENUS *PSEUDOMONAS*

Taxonomy

Pseudomonas is part of a large, heterogeneous and ubiquitous group of microorganisms generally referred to as pseudomonads. The pseudomonads are characterised as being highly metabolically versatile, bioactive, and prolific colonisers of surfaces. Pseudomonads are gram-negative, straight or slightly curved rods with polar flagella; they are chemo-organotrophs with a respiratory, non-fermentative type of metabolism and are usually catalase and oxidase-positive. The taxonomy of the group has been clarified using 16S ribosomal RNA sequence analysis (Table 1, Section IV).

The genus *Pseudomonas* corresponds to rRNA group I (Table 1). The type species for the genus is *Pseudomonas aeruginosa*. Strains are metabolically diverse, as well as having the capacity for denitrification and arginine degradation under anaerobic conditions. *P. aeruginosa* has been studied in more detail than any other pseudomonad using genetic techniques. Physical and genetic chromosome maps have been described (Romling et al., 1989; Ratnaningsih et al., 1990).

Applications

Pseudomonads have been identified to be of importance in bioremediation as a result of their tremendous capacity for biodegradation. They also offer considerable promise in agronomic applications, since many strains are bioactive, fast-growing, prolific colonisers of plant surfaces and are able to suppress or out-compete pathogenic and other deleterious microorganisms.

Pseudomonads as candidates for bioremediation

Nutritional versatility is exhibited widely amongst the pseudomonads. Combined with the presence or acquisition of catabolic plasmids by large numbers of strains, pseudomonads have the potential to mineralise a wide range of natural organic compounds, including aromatic hydrocarbons. This versatility allows for the rapid evolution of new metabolic pathways for the degradation of synthetic compounds (xenobiotics), leading to their complete oxidation and mineralisation. The complexity of the catabolic routes indicates sophisticated systems of regulation to control the expression and achieve the co-ordination of catabolic activities. Although the degradative pathways of pseudomonads vary considerably, the metabolic routes are convergent and lead to a limited number of common intermediates such as catechols. These represent key intermediates for aromatic compound degradation.

It is also anticipated that the nutritional versatility of pseudomonads and the application of molecular genetic techniques will be harnessed in the design of catabolic pathways for environmental purposes (Ensley, 1994; Timmis, 1994). For example, a *Pseudomonas* strain was recently isolated that can utilise TNT (2,4,6-trinitrotoluene) as a sole nitrogen source, producing toluene, aminotoluene and nitrotoluenes as end products. This organism was, however, unable to utilise toluene as a carbon source for growth. By introducing the entire toluene degradation pathway carried on the TOL plasmid pWWO-Km, an organism was produced that could potentially completely mineralise TNT (Ensley, 1994). Despite some of the TNT being completely mineralised, the formation of some dead-end metabolites by reduction of the nitrotoluenes to aminotoluenes remains a problem.

Agronomic applications

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. Furthermore, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These traits include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to compete aggressively with other microorganisms for niches and to exclude phytopathogens.

SECTION III – FORMAT OF THE INFORMATION IN THIS CONSENSUS DOCUMENT

The information format presented in this section is based on the ***General Scientific Considerations***, ***Human Health Considerations*** and ***Environmental and Agricultural Considerations*** from the OECD "Blue Book" (OECD, 1986) (see the Appendix to this consensus document). These Considerations were also used as the reference point in Environment Monograph No. 100, *Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology* (OECD, 1995), which identified commonalities among OECD countries with respect to data elements used during regulatory assessments.

It was decided at a meeting of the OECD Task Group on Environmental Applications of Modern Biotechnology in October 1995 that, for the purpose of producing consensus documents containing information for use during regulatory assessments, a subset of the considerations addressed in the OECD "Blue Book" would be appropriate. The subset presented here reflects the removal of considerations in the "Blue Book" that were duplicative or were possibly ambiguous in meaning:

Information Elements

A. General Considerations

Taxonomy, identification, source, culture

1 Subject of document: species included and taxonomic considerations;

2 Characteristics of the organism which permit identification, and the methods used to identify the organism;

3 Information on the recipient organism's reproductive cycle (sexual/asexual);

4 Biological features and environmental conditions which affect survival, reproduction, growth, multiplication or dissemination;

5 Behaviour in simulated natural environments such as microcosms, growth rooms, greenhouses, insectaries, etc.;

6 History of use (examples of environmental applications of the organism and information derived from these examples);

Genetic characteristics of the organism

7 Characterisation of the genomes (e.g. presence of large plasmids, insertion sequences), and stability of these characteristics;

8 Genetic transfer capability;

B. Human Health Considerations

Characteristics of the organism

9 Diseases caused and mechanism of pathogenicity, including invasiveness and virulence;

10 Communicability;

11 Infective dose;

12 Host range, possibility of alteration;

13 Capacity for colonisation;

14 Possibility of survival outside of human host;

15 Means of dissemination;

16 Biological stability;

17 Antibiotic-resistance patterns;

18 Toxigenicity;

19 Allergenicity;

20 Availability of appropriate prophylaxis and therapies;

C. Environmental and Agricultural Considerations

Ecological Traits of the Organism

21 Natural habitat and geographic distribution. Climatic characteristics of original habitats;

22 Significant involvement in environmental processes, including biogeochemical cycles, and potential for production of toxic metabolites;

23 Pathogenicity - host range, infectivity, toxigenicity, virulence, vectors;

24 Interactions with and effects on other organisms in the environment;

25 Ability to form survival structures (e.g. spores, sclerotia);

26 Routes of dissemination, physical or biological;

Application of the Organism in the Environment

27 Containment and decontamination;

28 Description of detection and monitoring techniques, including specificity, sensitivity and reliability.

The information elements numbered 1-28 above were adopted as the framework for producing this* Pseudomonas *consensus document.

In Section IV, each of these information elements has been used as a prompt to collate the information in the scientific literature which is applicable to the assessment of the environmental application of the microorganisms. The information is that available in the literature as of 30 June 1995. The literature search covered a number of databases. The literature search for human health information elements (9-20) covered Medline, Biosis, C.A.B., Embase, and Food Science and Technology Abstracts, back to 1966 or when the particular electronic database was established. Other information elements (4-6, 21, 23-24) grouped information by species. The literature search for these elements covered the following databases: *P. aeruginosa* (C.A.B. Abstracts, 1972-June 1995; B.C.I., 1991-June 1995; Chapman and Hall CD-ROM, 1995); *P. chlororaphis* (and *P. aureofaciens*) (C.A.B. Abstracts, 1972-June 1995; B.C.I., 1991-June 1995; Agricola, 1970-1995; DNP CD-ROM, 1995); *P. fluorescens* (and *P. marginalis*) (C.A.B. Abstracts, 1979-June 1995); *P. fragi* (C.A.B. Abstracts, 1972-June 1995; B.C.I., 1991-June 1995); *P. putida* (C.A.B. Abstracts, 1979-June 1995); *P. syringae* (C.A.B. Abstracts, 1972-June 1995; B.C.I., 1991-June 1995); and *P. tolaasii* (C.A.B. Abstracts, 1972-June 1995; B.C.I., 1991-June 1995).

The information is restricted to that available for the following seven species of the genus *Pseudomonas*: *P. aeruginosa*, *P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi*, *P. putida*, *P. syringae*, and *P. tolaasii*.

Further, the information is restricted to that available for the naturally occurring species; information on any genetically modified strains is excluded unless it bears directly on the properties of the naturally occurring organism.

SECTION IV – INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING *PSEUDOMONAS*

A. General Considerations

1. Subject of the document: species included and taxonomic considerations

1.1 Species included

The subject of this document is a subset of seven species within the genus *Pseudomonas*, most of which produce fluorescent pigments. Many members of this set have been, or are likely to be, employed in various biotechnological applications in the environment. The seven species are: *P. aeruginosa, P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi, P. putida, P. syringae,* and *P. tolaasii.*

1.2 Taxonomic considerations

1.2.1 The genus Pseudomonas

Prior to 1973, *Pseudomonas* was seen as one large heterogenous genus with members sharing a few phenotypic features. Palleroni et al. (1973) concluded that five groups approximating genera, which were established on the basis of rRNA sequence homologies (Table 1), appeared phylogenetically distant from each other. Though these groupings were confirmed through DNA hybridisation experiments (Johnson and Palleroni, 1989), it nonetheless took a decade to transform them into discrete taxonomic units based on both phenotypic and genotypic associations. The groupings now comprise units of larger than genus rank. Species once called *Pseudomonas* are now classified as members of at least a dozen genera found within the original five homology groups (Table 1; Yabuuchi and De Vos, 1995a; Yabuuchi and De Vos, 1995b). The genus *Pseudomonas* is now strictly confined to members of the rRNA group I (Table 1).

The members of this genus still represent a somewhat heterogenous collection of bacteria, but they are far more closely allied to each other than they are to species formerly having the genus name *Pseudomonas*. The type species for the genus is *Pseudomonas aeruginosa*. Strains of *P. aeruginosa* can be isolated from many environmental substrates, and appear uniform in a number of diagnostic characters (Palleroni, 1992b). It can be argued that *P. fluorescens* is more "typical" of the genus than is *P. aeruginosa*, but due to the difficulty of establishing defining characteristics for *P. fluorescens*, *P. aeruginosa* remains the choice for the type species (Palleroni, 1992c).

Common characteristics of the genus* Pseudomonas*:

- gram-negative
- rod-shaped (straight, asporogenous, 0.5-1.0 X 1.5-4.0 μm)
- motile due to polar flagella
- oxidase-positive (except for *P. syringae*)
- oxidative metabolism (mostly saccharolytic, some non-saccharolytic species, no gas formation from sugars)

- chemo-organotrophs
- catalase-positive
- growth with acetate as sole carbon source, most non-fastidious, few require growth factors
- NO_3 reduced to NO_2 or molecular N_2
- accumulate longer-chained polyhydroxyalkanoates
- produce pigments
- indole-negative

1.2.2 The "fluorescent" subgroup

The seven species considered in this document [*P. aeruginosa*, *P. chlororaphis* (including *P. aureofaciens*), *P. fluorescens* (including *P. marginalis*), *P. fragi*, *P. putida*, *P. syringae*, and *P. tolaasii*] are considered to be closely related to each other except for *P. aeruginosa* and *P. syringae* (Molin and Ternström, 1986; Janse et al., 1992). These seven species are considered as the fluorescent subgroup of the rRNA group I, although *P. fragi* includes non-fluorescent strains.

P. fluorescens, *P. putida* and *P. chlororaphis* are seen as forming a complex, intertwined by a continuum of transitional strains (Molin and Ternström, 1986; Barrett et al., 1986). Complicating the classification scheme is the observation that both *P. fluorescens* and *P. putida* comprise several biovars, each of which may deserve species rank, but which are so interconnected that adequate methods have not been devised to clearly separate the member strains for each biovar. *P. chlororaphis*, which also encompasses the strains formerly called *P. aureofaciens* and *P. lundensis*, a recently described species, were once considered to belong to separate biovars of *P. fluorescens*.

Also closely associated with the *fluorescens-chlororaphis-putida* complex is *P. fragi*. This species has some fluorescent strains, but is primarily non-fluorescent. *P. fragi* is also a complex of different phenotypes, many of which are closely allied with some biovars of *P. fluorescens* and also could be misidentified as *P. putida* (Molin and Ternström, 1986).

P. aeruginosa, the type species and most clearly defined member of the genus, is seen as separate from the *fluorescens-chlororaphis-putida* complex.

P. syringae and *P. tolaasii* are pathogens in a group that also includes other pathogenic species (e.g. *P. cichorii* and *P. viridiflava*). However, *P. tolaasii* is an oxidase-positive mushroom pathogen related to, and potentially confused with, members of *the P. fluorescens* supercluster (Janse et al., 1992). *P. syringae* is an oxidase-negative plant pathogen comprising many pathovars derived from taxa that previously had species rank (Palleroni, 1984).

Fluorescence

Pigments often provide valuable diagnostic characters, since their production invariably correlates well with other group properties. Fluorescent pigments are produced abundantly in media with a low iron content; fluorescence varies from white to blue-green upon excitation with ultraviolet radiation. King's medium B is frequently used for the isolation of pseudomonads, especially by plant pathologists (King et al., 1954). Fluorescent species of *Pseudomonas* produce pyoverdin and/or phenazine pigments. Pyoverdin production is characteristic of most species. Palleroni (1984) indicates that *P. fluorescens* biovars II and V, along with *P. chlororaphis* and *P. putida* biovar B, have variable (11-89% positive) pyoveridin production. Although positive pyocyanin production is diagnostic for *P. aeruginosa*, the reverse is not necessarily true.

Table 1

Phylogeny and current classification of the pseudomonads

Proteobacteria subclass	rRNA group	Original name	Current classification	Characteristics
Gamma	I	* *P. aeruginosa*	*Pseudomonas*	type species; opportunistic pathogen
		* *P. fluorescens*		fluorescent supercluster; oxidase positive, mostly fluorescent, saprophytic or opportunistic pathogens
		* *P. chlororaphis*		
		P. lundensis	* *P. chlororaphis*	name reclassified
		* *P. putida*		
		* *P. tolaasii*		mushroom pathogen
		P. marginalis	* *P. fluorescens*	name reclassified
		P. aureofaciens	* *P. chlororaphis*	name reclassified
		* *P. fragi*		some strains non-fluorescent
		* *P. syringae*		fluorescent, plant or mushroom pathogen. *P. syringae* and *P. viridflava* are oxidase-negative. *P. syringae* comprises many pathovars
		P. viridiflava		
		P. cichorii		
		P. agarici		
		P. asplenii		
		P. flavescens		fluorescent
		P. alcaligenes		non-fluorescent
		P. citronella		
		P. mendocina		

(continued on next page)

Table 1

Phylogeny and current classification of the pseudomonads (cont.)

		P. oleovorans		
		P. pseudoalcaligenes		
		P. stutzeri		
Gamma	V	*P. maltophila*	*Stenotrophomonas maltophilia*	related to *Xanthomonas*
		P. marina	*Delaya marina*	
Beta	III	*P. acidovorans*	*Comamonas*	
		P. terrigena		
		P. testosteroni		
		P. avenae	*Acidovorax*	facultatively autotrophic species (hydrogen pseudomonad)
		P. delafieldii		
		P. facials		
		P. flava	*Hydrogenophaga*	facultatively autotrophic species (hydrogen pseudomonad)
		P. palleroni		
		P. pseudoflava		
		P. taenispiralis		
		P. saccharophila	*Pseudomonas*	
		P. ruhlandii	*Alcaligenes xylosoxidans*	

Table 1

Phylogeny and current classification of the pseudomonads (cont.)

Beta	II	*P. cepacia*	*Burkholderia*	heterogenous genus
		P. caryophylli		
		P. gladioli		
		P. mallei		
		P. pickettii		
		P. pseudomallei		
		P. solancearum		
Alpha	IV	*P. paucimobilis*	*Sphingomonas paucimobilis*	
		P. carboxidovorans	*Oligotropha carboxidovorans*	
		P. aminovorans	*Aminobacter aminovorans*	
		P. mesophilica	*Methylobacterium mesophilicum*	
		P. sp.	*Chelatobacter heintzii*	
		P. compransoris	*Zavarzinia compransoris*	
		P. diminuta	*Brevundimonas diminuta*	very distantly related to rRNA group I
		P. vesicularis	*Brevundimonas vesicularis*	

Compiled from Palleroni, 1992b and 1992c; Molin and Ternström, 1986; Yabuuchi et al., 1995a and 1995b, and Hildebrand et al., 1994. The species which are the focus of this document are indicated with an asterisk.

Plasmid-encoded characteristics

Plasmid-encoded characteristics such as antibiotic resistance, chemical resistance and metabolic capabilities are important components of the pseudomonad genome. Some examples, for the species under consideration, are given in Table 2. Many if not most of these characteristics are strain-specific and of little value in terms of taxonomy and identification. An exception to this rule, however, is phage susceptibility. Plasmid-encoded phage susceptibility can be important in differentiating *P. syringae* pathovars when combined with biochemical testing, and *P. aeruginosa* isolates have been typed to the subspecies level using phage sensitivity.

2. Characteristics of the organism which permit identification and the methods used to identify the organism

2.0 General considerations

P. aeruginosa is distinct and readily distinguished from other members of the genus, and the pathovars of *P. syringae* can be distinguished by determination of their host range.

Distinction of the other five species in the group *(P. fluorescens, P. chlororaphis, P. putida, P. tolaasii, P. fragi*) from each other is not straightforward, and the expression "continuum" is frequently used to describe their inter-relationship. Most authors agree that current methods are generally inadequate to ensure proper placement of new isolates within the related species *P. fluorescens*, and *P. putida* and their biovars (Palleroni, 1992b; Christensen et al., 1994; Barrett et al., 1986). Except for its pathogenicity, *P. tolaasii* is difficult to distinguish from *P. fluorescens* (Janse et al., 1992). *P. chlororaphis* is separable from *P. fluorescens* based on production of unique phenazine pigments (Palleroni, 1984), and has some distinctive substrate utilisation patterns (Barrett et al., 1986), but is otherwise well within the boundaries of the fluorescent supercluster (e.g. Janse et al., 1992). Finally, *P. fragi* shares many features with members of the fluorescent supercluster, but most strains are not fluorescent (Molin and Ternström, 1986).

2.1 Methods used for identification and classification

2.1.1 Numerical taxonomy

Numerical taxonomy has become the "traditional" method for classifying members of the genus *Pseudomonas*. This approach compares multiple features of the isolate, for which there is substantial discriminatory power, with a database of features of well-described members of the taxon. The accuracy of this type of approach will depend upon the quality and quantity of the data for strains comprising the reference database.

In order to achieve valid results, identical laboratory techniques need to be used for analysis of the isolate and the strains used to construct the reference database. The success of numerical taxonomy is also affected by the complexity of the relationships among the taxa being evaluated.

Use of a broad spectrum of substrates in numeric taxonomic evaluations has had some success for fluorescent species of *Pseudomonas* (e.g. Barrett et al., 1986). These techniques have permitted some assignment of strains to species and biovars within the fluorescent supercluster (a term applied to all of the species and biovars of *P. fluorescens, P. putida* and their allies, Table 1). However, Molin and Ternström

(1986) and Janse et al. (1992) both reported many unclassifiable strains among those they subjected to classical numeric taxonomic analyses.

Commercial suppliers have devised simplified, automated versions of this technique. Examples of commercial kits available for identification of *Pseudomonas* on the basis of carbon source utilisation patterns, and physiological and morphological characters, are the API20E (API, 200 Express Street, Plainview, New York 11803, USA; BioMerieux, F-69280 Marcy-L'Etoile, France) and the BIOLOG (BIOLOG Inc., Hayward, California, USA) systems. For these kits, the database for *Pseudomonas* is based on mainly clinical, not environmental, strains. As a result, the kits may fail to identify all environmental isolates.

Use of these kits requires experience. In addition, most of them are designed to determine the membership of the isolate within a taxon and not to distinguish strains within a species. That is, the test profile in most cases is not unique to a particular strain. So, in most cases, test profiles will not be sufficient to distinguish the isolate from other strains of the same species. If such a distinction is being made, it must be based on the detection of properties unique within the taxon.

Details of the test methodologies and profiles of the species can be found in Palleroni (1981; 1984; 1992c).

2.1.2 Genotypic approaches

The current classification of the pseudomonads is based on rRNA homologies. The variable and conserved regions of the RNA molecule are both important for identification purposes. The conserved regions serve as targets for polymerase chain reaction (PCR) primer binding sites and universal hybridization probes. The variable regions are the targets of the hybridization probes and primers that are taxon-specific. Probes and PCR primers directed at diagnostic rRNA sequences have facilitated the classification of pseudomonads into the five rRNA groups (Table 1).

Strong selection pressure for the conservation of 16S and 23S rRNA molecular structure and sequence has meant that rRNA molecules are powerful evolutionary clocks for describing phylogenetic relationships between rRNA groups of pseudomonads. At present, however, they are unable to position individual strains into species groups. This is particularly true for the fluorescent rRNA group I pseudomonads (Christensen et al., 1994). Using 23S rDNA methods, Christensen et al. (1994) found that "the method failed to provide a basis for distinguishing between *P. fluorescens, P. chlororaphis,* and *P. putida* Biovar B and to differentiate among the biovars of *P. fluorescens*." This study also showed that there did not seem to be a correspondence between taxonomies of this group based on 23S ribosomal sequences and from conventional numerical taxonomy. As pointed out by Janse et al. (1992), the large number of intermediate strains of all of these species shows "more variation than the present schemes (for classification) allow."

Schleifer et al. (1992) describe several probes for the rapid identification of members of the genus *Pseudomonas*. A 360 bp fragment of a 23S rRNA gene derived from *P. aeruginosa* (Festl et al., 1986) allowed differentiation of the eleven fluorescent and non-fluorescent group I species tested. A second probe was group-specific for *P. stutzeri, P. alcaligenes, P. pseudoalcaligenes* and *P. aeruginosa*. This probe comprises a 255 bp fragment of the 23S rDNA of *P. stutzeri* that is homologous to the bases 1366 to 1617 of *Escherichia coli* 23S rRNA. Both probes were tested by "dot-blot" hybridization against genomic DNA bound to filters.

Table 2

Examples of plasmids responsible for the metabolism of organic compounds or resistance to heavy metals in fluorescent *Pseudomonas* species

Organism	Plasmid	Substrate	Reference
P. putida	CAM	camphor	Jacoby, 1975
	TOL	xylene, toluene	White & Dunn, 1978
	SAL	salicylate	Korfhagen et al., 1978
	NAH	napthalene	White & Dunn, 1978
	pRE4	isopropyl-benzene	Eaton & Timmis, 1986
	pEG	styrene	Bestetti et al., 1984
	pCINNP	cinnamic acid	Andreoni & Bestetti, 1986
	pAC25	3-chloro-benzoic acid	Chatterjee & Chakrabarty, 1983
P. fluorescens	pQM1	mercury	Bale et al., 1988
P. syringae pv. *tomato*	pPT23a; pPT23c	copper	Bender & Cooksey, 1986

Schleifer et al. (1992) used fluorescein or tetramethylrhodamine 5'end-labelled sequences as probes to identify *Pseudomonas* species. The first probe (P72; 5'-TTCAGTACAAGATACCTA) differentiated *P. aeruginosa*, *P. alcaligenes* and *P. pseudo-alcaligenes* from the other group I species. A second probe, Ps (5'-GAAGGCTAGGCCAGC), identified all species except *P. putida*. An oligonucleotide specific for the *P. putida* sequence, 5'-GAAGGUUAGGCCAGC, allowed differentiation of *P. putida*, and a mixed probe (i.e. both oligonucleotides) allowed detection of all species of *Pseudomonas*.

Polymerase chain reaction (PCR)

P. aeruginosa strains can be identified by PCR-based amplification of the 16S-23S rDNA internal transcribed spacer region with specific primers (Tyler et al., 1995).

2.1.3 Other biomarkers

Biomarkers such as fatty acid methyl esters (FAMES) have been used widely for the identification of bacterial species (Thompson et al., 1993b). A commercial identification system, the Microbial Identification System (MIDI, Newark, New Jersey, USA), offers an extensive database of strains, including many pseudomonad strains, to which the fatty acid profile of an unknown isolate may be compared. The libraries of strains contain well-described clinical, environmental and plant-pathogenic strains. Tahis system provides an identification at the species level and a diagnostic profile; however, it is unlikely to identify all environmental isolates, since many have not been described before. Whole cell fatty acid analysis was tested as a method to discriminate between members of the *Pseudomonas* fluorescent supercluster (Janse et al., 1992). This analysis resulted in recognition of a large supercluster that included most *P. fluorescens* and related strains (*P. chlororaphis*, *P. putida* and *P. tolaasii*). In the supercluster there were no separate clusters discriminating biovars of *P. fluorescens*, the other related species, or strains received as *P. marginalis* (a name formerly applied to plant-pathogenic members of the supercluster). Thus, the resolution of this technique appears to have limitations.

Diagnostic profiles for microorganisms may also be obtained using polyacrylamide electrophoresis of whole cell protein extracts, or DNA fingerprints produced via restriction endonuclease digestion of genomic DNA. Pseudomonads have genomes that are rich in GC DNA bases. Enzymes like SpeI that cut at sites with a high AT base composition will digest the DNA at only a few sites, producing large fragments which may be separated and analysed using pulse field gel electrophoresis.

The species of rRNA group I synthesize a ubiqionone with nine isoprene units (Q-9) in the side chain, whereas members of rRNA groups II, III, and V contain Q-8, and those of rRNA group IV a Q-10 (Oyaizu and Komgata, 1983).

Polyamine patterns are of similar utility: rRNA group I species have a high putrescine and spermidine content, rRNA group II and III species have 2-hydroxuputrescine and a high content of putrescine, rRNA group IV species only contain significant amounts of spermidine and sym-homospermidine, and rRNA group V species are characterised by high concentrations of cadaverine and spermidine (Busse and Auling, 1988; Auling, 1992; Yang et al., 1993).

In summary, the results of any of the methods of identification described above are only as good as the database of strains and isolates to which they are referenced. There are numerous techniques that, if applied at the optimum taxonomic level, may prove useful in identifying *Pseudomonas* and its species. Ribosomal RNA sequencing seems useful at the genus or higher level, and methods like fatty acid analysis can work at the strain and isolate level.

3. Information on the reproductive cycle (sexual/asexual)

Pseudomonas species reproduce by cell growth and binary cell division.

4. Biological features and environmental conditions which affect survival, reproduction, growth, multiplication or dissemination

4.0 General considerations

Pseudomonas species are efficient saprophytic chemo-organotrophs which grow at neutral pH and at temperatures in the mesophilic range (optimal growth between 20° and 45°C). Some species will grow at 4°C (*P. fluorescens, P. putida)* but not at the elevated temperature of 41°C. Optimal temperatures are *25*-30°C for *P. fluorescens, P. putida and P. syringae*, 30°C for *P. chlororaphis,* and 37°C for *P. aeruginosa.* Most, if not all, species fail to grow under acid conditions (< pH 4.5) (Palleroni, 1984).

Pseudomonas species thrive under moist conditions in soil (particularly in association with plants), and in sewage sediments and the aquatic environments. Environmental conditions which will affect their growth include nutrient availability, moisture, temperature, predation, competition, UV irradiation, oxygen availability, salinity, and the presence of inhibitory or toxic compounds. As nutritional demands are modest, pseudomonads can survive and multiply for months in moist environments such as tap water, sink drains, etc. (Palleroni, 1981; Bergen, 1981).

Competition and competitive niche exclusion are likely to limit the growth of introduced pseudomonad inoculants. Competitors are likely to include closely related pseudomonads and other bacteria able to compete for the same ecological niches with similar nutritional requirements (Lindow, 1992).

A number of environmental conditions may affect the dissemination of *Pseudomonas* species including surface water runoff, wind currents, and rain splash. It is likely that insects and other animals may also serve as vectors for dispersal. For example, *P. aeruginosa* can inhabit part of the normal gut or skin microflora of humans and animals. Thus dissemination would be associated with these vectors.

4.1 *P. aeruginosa*

P. aeruginosa is capable of growing in conditions of extremely low nutrient content (Palleroni 1984). The species was found to survive and proliferate in water for up to 100 days or longer (Warburton et al., 1994). Conditions of high humidity and temperature (80-90% humidity, 27°C) favoured the colonisation of lettuce and bean plants (Green et al., 1974).

4.2 *P. chlororaphis*

P. aureofaciens (*P. chlororaphis*) is an important coloniser of the rhizosphere and phyllosphere of plants (Thompson et al., 1993a; Legard et al., 1994). Kluepfel et al. (1991b) reported the colonisation of wheat roots in a field release of a recombinant *P. aureofaciens* (*lac*ZY). This population of *P. aureofaciens* reached a maximum of $2x10^6$ cfu/g root two weeks after inoculation and declined steadily to reach a level below detection (<100 cfu/g root) by 38 weeks post-inoculation. Angle et al. (1995) found that inoculations of recombinant *P. aureofaciens* (*lac*ZY) survived approximately twice as long in wheat rhizosphere as in non-rhizosphere bulk soil.

England et al. (1993) compared the survival and respiratory activity of *P. aureofaciens* in sterile and non-sterile loam and sandy loam soil microcosms. Recovery of *P. aureofaciens* was greater in sterile than non-sterile soils. Respiratory activity was higher in sandy loam soil than in loam soil, but soil type had no effect on survival.

The growth of *P. aureofaciens* in the spermosphere of seed-inoculated sugarbeets exhibited long lag phases (8-12 h) and their populations increased mainly between 12 and 24 hours (Fukui et al., 1994). The doubling time during the exponential growth phases was 2-3 h (Fukui et al., 1994).

4.3 *P. fluorescens*

P. fluorescens is commonly found inhabiting plant rhizosphere or phyllosphere environments. The plant rhizosphere provides an environment in which the species may show improved survival and growth. *P. fluorescens* distributed homogeneously in soil can result in significantly higher numbers in the rhizosphere of young wheat plants than in non-rhizosphere soil (Trevors et al., 1990).

The survival of *P. fluorescens* is affected by a variety of abiotic and biotic factors. Rattray et al. (1993) found that temperature and soil bulk density had a significant effect on lux-marked *P. fluorescens* colonisation of wheat rhizospheres. The greatest rates of colonisation occurred at the highest temperature (22°C) and lower bulk density (0.82 g/cm^3), and 100-fold higher numbers were found in the ectorhizosphere than in the endorhizosphere. Van Elsas et al. (1991) found *P. fluorescens* cells were able to withstand low temperatures, and could survive better at 4°C than at 15 or 27°C following introduction into natural soil, possibly due to an inhibition of the activity of the indigenous microflora. Van Elsas et al. (1986) found that *P. fluorescens* cell numbers declined slowly in both silt loam and loamy sand, but survival was better in the silt loam. Heijnen et al. (1993) found that *P. fluorescens* survived better in unplanted soils in the presence of bentonite clay. Stutz et al. (1989) demonstrated that survival of *P. fluorescens* in vermiculite clay was better than in montmorillonite, which was better than in illite.

Van Elsas et al. (1992) found *P. fluorescens* R2f survived above 10^7 cfu/g dry soil for up to 84 days in Ede loamy sand microcosms when encapsulated in alginate with skim milk and bentonite clay, while free cells declined below 10^5 cells/g dry soil after 21 days. Vandenhove et al. (1991) studied the survival of *P. fluorescens* inocula of different physiological stages in soil. Introduction of a late exponential phase inoculum into soil brought about a lower death rate compared to exponential or stationary phase inocula.

Handley and Webster (1993) studied the effect of relative humidity (RH at 20, 40, 60, and 80%) on airborne survival of *P. fluorescens* indoors. They found that *P. fluorescens*, suspended in distilled water, survived best at mid humidities and least at 80% relative humidity.

Boelens et al. (1994) and Bowers and Parke (1993) determined that motility of *P. fluorescens* did not affect its spread through soil. A non-motile mutant strain promoted plant growth and colonised roots as effectively as the motile strain. Water flow rates were more important than motility for dispersal through soil and rhizospheres. Knudsen (1989) developed a mathematical model for predicting aerial dispersal of bacteria during environmental release which predicted off-site dispersal patterns that were in qualitative agreement with results from a field release of a genetically engineered *P. fluorescens* in California.

Thompson et al. (1992) studied dissemination of *P. fluorescens* by placing bacterial populations on apple or pear pollen in the entrances of hives of honey bees. In a pear orchard, 72% of the flowers within 7.6 m of the hive were colonised with *P. fluorescens* eight days after the start of the study.

4.4 *P. fragi*

P. fragi is commonly found on refrigerated meat and dairy products (Jay, 1992). Psychrotrophs such as *P. fragi* generally have a lower metabolic rate than mesophiles (lower Q_{10} for the same substrate) and have membranes that transport solutes more efficiently (Jay, 1992). In addition, there is a correlation between the maximum growth temperature and the temperature at which respiratory enzymes are destroyed in psychrotrophs. Nashif and Nelson (1953) reported that extracellular lipase synthesis in *P. fragi* was inactivated at 30°C. The lipase of *P. fragi* is reported active at temperatures as low as -29°C (Alford and Pierce, 1961).

P. fragi has the ability to colonise stainless steel surfaces in food processing establishments to form "biofilms" (Hood and Zottola, 1995); attachment may involve a polysaccharide and protein matrix surrounding the cells (Herald and Zottola, 1989). Attachment of *P. fragi* to stainless steel surfaces occurred in 0.5 h at 25°C and in 2 h at 4°C through the development of attachment fibrils (Stone and Zottola, 1985).

4.5 *P. putida*

A variety of environmental factors can affect the survival of *P. putida.* For example, plant rhizospheres can provide an environment for improved survival. Gamliel and Katan (1992) studied the chemotaxis response of *P. putida* towards seed exudates and germinating tomato seeds and suggested this may contribute to its rapid establishment in plant rhizospheres. Temperature is also an important factor. Hartel et al. (1994) found that *P. putida* (*lac*ZY) declined from about 10^8 to 10^3 cfu/g of soil after 35 days at 35°C, while it did not survive after three days at 40°C.

Macnaughton et al. (1992), using pLV1013 as a marker plasmid in *P. putida* PaW8, investigated the effect of soil texture on survival and found that introduced bacteria survived better in soils with higher clay content. Compeau et al. (1988) studied survival of *P. fluorescens* and *P. putida* strains in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there were limited sites for colonisation of *Pseudomonas* species in these soils.

Lynch (1990) found that *P. putida* WCS 358 survived in distilled water (without substrate input) for over a year. It was suggested that this could result from the utilisation of dead cells within the population, and from viable cells having a very low maintenance energy requirement in a state of arrested metabolism.

Madsen and Alexander (1982) found that cells of *P. putida* were not transported below 2.7 cm in moist soil in the absence of some transporting agent or in the presence of developing plant roots. Percolating water and a burrowing earthworm enhanced the vertical transport of *P. putida*.

4.6 *P. syringae*

Foliar plant pathogens such as *P. syringae* are generally not adapted to survival in the soil (Lindow et al., 1988). However, *P. syringae* has been isolated from plant debris in the soil and can overwinter in temperate climates (Hirano and Upper, 1983). *P. syringae* pv. *syringae* R32 have pili that function as adhesions anchoring the cell to the surface of plants, thereby enhancing epiphytic colonisation.

Wild-type bacteria became virtually resistant to displacement by rinsing within one day after inoculation, whereas non-pilated mutants were only partially resistant within three days (Suoniemi et al., 1995).

P. syringae metabolises a broad range of substrates, thus demonstrating flexibility in nutrient utilisation (Hirano and Upper, 1990). Criteria for viability of cells have been modified as the result of starvation experiments with *P. syringae*; it was determined that respiration of acetate and glycerol were more accurate determinants of viability than respiration of succinate (Cabral, 1995). The use of bactericides in agriculture (streptomycin and copper) has resulted in selection for strains resistant to these compounds; the resistance is often encoded on plasmids (Cooksey, 1990).

Plant-pathogenic strains grow to larger population sizes on susceptible plant hosts than on resistant ones (Stadt and Saettler, 1981), and therefore pathovars of *P. syringae* will grow to greater numbers on their respective hosts than on non-hosts. The presence of free water may be the most important factor contributing to the increase in population of *P. syringae* pv. *syringae* to infectious levels on bean leaves (Hirano and Upper, 1983; 1990; Beattie and Lindow, 1994). Immediately after rainfall, there is an initial decrease in population as bacteria are washed off the leaf surface, followed by a rapid increase in the population within 12 to 24 hours. Ambient temperature appears to have little effect on field populations of *P. syringae* pv. *syringae* on leaves but the age of annual crops does have an effect, with many more cells found on older leaves than on younger ones (Hirano and Upper, 1990; Jacques et al., 1995). *P. syringae* pv. *savastanoi* causes tumors on olive and oleander by producing the plant growth regulators indoleacetic acid (IAA) and cytokinins following infection; mutants deficient in IAA production grew as well as the wild type in culture and on plants, but the wild type reached a higher population density and maintained its maximum density at least nine weeks longer than the mutant populations.

Rainfall plays an important role in redistributing *P. syringae* within the plant canopy by washing bacteria from upper leaves onto lower ones, and by allowing individual bacterial cells to move using their flagella and find protected micro-sites on the surface of the leaf (Beattie and Lindow, 1994). Rainfall efficiently removed bacteria from foliar surfaces, but most of the cells were washed onto the soil; only a small portion were washed a relatively short distance from the source (Butterworth and McCartney, 1991).

. *P. syringae* is also dispersed on seeds (Hirano and Upper, 1983). When cells of *P. syringae* were applied as a spray to plots, an exponential decrease in numbers of cells was observed; some cells were detected 9.1 m downwind within 20 minutes of the spray application. When applied to oat plants (a non-host), viable cells could be detected for up to 16 days and were detected on plants up to 27 m downwind. In contrast to the plants, viable cells could not be detected in the upper layers of soil after two days (Lindow et al., 1988).

4.7 *P. tolaasii*

In the production of commercial mushrooms, *P. tolaasii* probably survives between crops on structural surfaces, in debris, and on equipment. It can be moved readily from one crop to another on the hands of pickers, on materials or equipment used in harvesting, and by insects, mites, water droplets and mushroom spores.

Conditions of high relative humidity and surface wetness encourage the expression of symptoms of brown blotch, an important mushroom disease, caused by *P. tolaasii.* Dispersal of the microorganisms occurs readily upon watering once the disease is established (Howard et al., 1994).

5. Behaviour in simulated natural environments such as microcosms, growth rooms, greenhouses, insectaries, etc.

5.1 *P. aeruginosa*

In a study by Sturman et al. (1994), the growth rate of *P. aeruginosa* appeared to be important in determining interspecies competition within packed-bed bioreactors filled with diatomaceous earth pellets.

5.2 *P. chlororaphis*

Angle et al. (1995) found that an intact soil core microcosm closely simulated survival results obtained from a field release of a recombinant *P. aureofaciens* (*lac*ZY). The strain of *P. aureofaciens* survived approximately 63 days in the bulk soil microcosm and 96 days in the rhizosphere microcosm.

5.3 *P. fluorescens*

Binnerup et al. (1993) found that kanamycin-resistant cells of *P. fluorescens* DF57-3 (Tn5 modified) inoculated in soil microcosms rapidly lost their culturability, as defined by visible colony formation on Kings B agar supplemented with kanamycin. After 40 days, only 0.02 to 0.35% of the initial inoculum was culturable. It was determined that about 20% of the initial inoculum represented viable, but non-culturable cells.

Compeau et al. (1988) studied survival of *P. fluorescens* and *P. putida* in sterile and non-sterile soil. Colonisation of sterile soil by one strain precluded normal colonisation of the second added strain and suggested there are limited sites for colonisation of *Pseudomonas* species in these soils. Similarly, Al-Achi et al. (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested that there was competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

5.4 *P. fragi*

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

5.5 *P. putida*

Winstanley et al. (1993) studied the survival of *P. putida,* with an xy/E marker insert, in soil and lake water microcosms. When released into these microcosms, populations of the marked *P. putida* had a steady decline with little or no apparent division of cells. The rate of decline of *P. putida* in soil microcosms was significantly greater at 35% than at 50% field capacity water content, indicating that water content of the soil had an effect on survival. Similarly, Iwasaki et al. (1993; 1994) reported that the density of *P. putida* decreased rapidly to less than 10^2 to 10^3 level within five days in lake water and soil microcosms. The survival was influenced by protozoa density, light intensity, and soil water content. The addition of *P. putida* (10^7 cfu/ml) into natural water and soil had no effect on the density of indigenous microorganisms and enzyme activities.

Williams et al. (1992) studied the fate and effects of *P. putida* PPO200 genetically engineered for both nalidixic acid and kanamycin resistance in freshwater and marine microcosms. The freshwater microcosm contained fish (*Poecilia latipinna*), annelid worms (*Tubifex tubifex*), snails (*Gyraulus* sp.), freshwater mussels (*Margaritifera margaritifera*), freshwater shrimps (*Palaemonetes kadiakensis*), and plants (*Elodea canadensis*). The marine microcosm contained fish (*Cyprinodon variegatus*), sea anenomae (*Bunodosoma californica*), snails (*Turbo fluctosus*), oysters (*Crassostrea gigas*), estuarine shrimp (*Palaemonetes pugio*), shorefly larvae (*Ephydra* sp.), and plants (*Salicornia biglovii*). *P. putida* could be detected in the tissues of some non-target organisms, i.e. the bacterium survived. However, gross signs, survival, and the histological study of control and exposed non-target organisms indicated that there were no adverse effects.

Doyle et al. (1991) and Short et al. (1991) observed reductions in CO_2 evolution and the number of fungal propagules, as well as the enhancement of dehydrogenase activity in soil amended with 2,4-dichlorophenoxyacetate (2,4-D) and inoculated with *P. putida* PPO301(pRO103) genetically engineered to degrade 2,4-D. These unanticipated effects were not observed: (a) in uninoculated soil; (b) when the homologous, plasmidless parent *P. putida* PPO301 was inoculated; or (c) in the presence of the genetically engineered *P. putida* when 2,4-D was not added. Moreover, the effects were not predictable from the phenotype of this genetically engineered *P. putida*. While long-term, statistically significant differences were detected in some microbial populations and processes, the majority of the differences were transient.

The effects of *P. putida*, on nitrogen transformations and nitrogen-transforming microbial populations were studied in a soil perfusion system by Jones et al. (1991). Neither the genetically engineered strain nor its homologous plasmidless host had a significant effect on ammonification, nitrification or denitrification in the soil, or on the population dynamics of the microorganisms responsible for these processes.

5.6 *P. syringae*

Wendtpotthoff et al. (1994) monitored the fate of a genetically engineered strain of *P. syringae* applied to the leaves of bush beans in a planted soil microcosm. *P. syringae* established on the bean leaves at between 5×10^3 and 4×10^6 cfu/gm^{-1} fresh weight. During senescence of the bean plants, the strain was no longer detectable by selective cultivation and subsequent colony hybridization.

Significant differences within *P. syringae* strain MF714R were detected when the bacterium was cultured on agar or in broth or collected from colonised leaves and subsequently inoculated onto greenhouse-grown plants in growth chambers or in the field or onto field grown plants. Bacterial cells cultured in liquid medium survived the least well after inoculation under all conditions, whereas cells cultured on solid media exhibited the highest percent survival and desiccation tolerance in the growth chamber but survived less well in the field than did cells harvested from plants. Cells harvested from plants and inoculated onto plants in the field usually had the highest percent survival, started to increase in numbers earlier, and reached a higher number than did cells cultured *in vitro* (Wilson and Lindow, 1993a).

Wilson and Lindow (1993b) indicated that greenhouse-grown plants support larger epiphytic populations of an inoculated strain of *P. syringae* than do field-grown plants.

5.7 *P. tolaasii*

No information was found regarding the behaviour of this species in microcosms, greenhouses, insectaries, etc.

6. History of use (examples of environmental applications of the organism and information derived from these examples)

6.0 General considerations

Pseudomonads have been identified to be of importance in bioremediation as a result of their metabolic versatility. This metabolic versatility, and the ability to acquire additional versatility via plasmids, provides the potential for the rapid evolution of novel metabolic ability in *Pseudomonas* species. Examples of useful, or potentially useful, environmental applications of *Pseudomonas* isolates are given in Table 3. Some pseudomonad species have been introduced into the environment in bioremediation studies and have provided valuable information pertaining to characteristics such as survival. For example, Thiem et al. (1994) injected *Pseudomonas* sp. strain B13, a chlorobenzoate degrader, into a subsurface aquifer and found they could detect the strain 14.5 months after its environmental introduction.

Pseudomonads also have great potential in agronomic applications, since they are prolific colonisers of plant surfaces and represent a significant component of plant microflora. For example, they have been identified to possess traits that make them suitable as agents for biological pest control (O'Sullivan and O'Gara, 1992). These include an ability to produce antimicrobial molecules (antibiotics, antifungals and siderophores) and a capacity to aggressively compete with other microorganisms for niches and exclude phytopathogens. The possibility of the environmental application of strains to minimize frost damage on crop plants has also been investigated (Lindow and Panopoulos, 1988).

6.1 *P. aeruginosa*

Some strains of *P. aeruginosa* have been shown to produce biosurfactants which have potential uses in bioremediation for washing hydrocarbons from soil (Van Dyke et al., 1993). Jain et al. (1992) found that biosurfactants produced by *P. aeruginosa*, when added to soil, significantly enhanced the degradation of tetradecane, hexadecane and pristane.

Degradation of pentachlorophenol by *P. aeruginosa* has been investigated in shake-cultures. The bacteria were able to completely degrade pentachlorophenol up to 800 mg/l in six days with glucose as a co-substrate (Premalatha and Rajakumar, 1994). *P. aeruginosa* has also been found to degrade styrene in a continuous reactor at a rate of 293 mg $g^{-1}h^{-1}$. This could be applied to the industrial treatment of waste gas or polluted water (El Aalam et al., 1993).

6.2 *P. chlororaphis*

A strain of *P. chlororaphis,* genetically engineered to contain the *lacZY* genes, was introduced into the environment in a field trial in the United States in 1987, and its behaviour compared to the non-engineered strain (Kluepfel et al., 1991a, b, c). The non-engineered strain increased in number for two weeks, then declined to at or near the detection limit by 31 weeks.

Table 3

Examples of fluorescent species of *Pseudomonas* reported to have been used, or to have potential use, for bioremediation

Species	Strain	Target chemical	Reference
P. aeruginosa	JB2	halogenated benzoic acids	Hickey & Focht, 1990
	PaK1	polyaromatic hydrocarbons	Kiyohara et al., 1994
P. fluorescens	PHK	phthlate	Pujar & Ribbons, 1985
		dimethylphenol	Busse et al., 1989
		isopropylbenzene	Busse et al., 1989
P. putida		methyl-benzoates	Galli et al., 1992
		napthalene sulphonic acid	Zurrer et al., 1987
		dimethylphenol	Busse et al., 1989
	OUS82	polyaromatic hydrocarbons	Kiyohara et al., 1994
	G7	polyaromatic hydrocarbons	Kiyohara et al., 1994

6.3 *P. fluorescens*

P. fluorescens has been genetically engineered and used in a number of experimental field studies, including the environmental introduction of a Tn-5 marked strain in the Netherlands in 1986 and other studies in the United States with strains engineered by deletion of the ice gene, and by introduction of *lacZY* marker genes (Wilson and Lindow, 1993b). De Leij et al. (1995) found that field releases of a genetically engineered *P. fluorescens*, and the unmodified wild-type strain, resulted in significant but transient perturbations of some of the culturable components of the indigenous microbial communities that inhabited the rhizosphere and phylloplane of spring wheat, but no significant perturbations of the indigenous culturable microbial populations in non-rhizosphere soil were found. The release of both of these bacteria had no obvious effect on plant growth and plant health, and the observed microbial perturbations were considered minor.

P. fluorescens can produce large and unusual proteins that are a key component of bacterial ice nuclei (Warren, 1987). Warren (1987) and Lindow and Panopoulos (1988) reviewed the practical applications connected to ice nucleation, including snow-making and the use of ice nucleation gene-deletion strains to generate biological control agents for minimizing frost damage to plants. A naturally occurring *P. fluorescens* strain, A506, has been registered commercially for the control of frost injury of pear (Wilson and Lindow, 1993b).

P. fluorescens is also one of the more common bacterial species that has been used for the control of diseases in the phyllosphere of plants, and a naturally occurring strain of this species has been registered for the commercial control of fire blight on pear (Wilson and Lindow, 1993b). Hatai and Willoughby (1988) detected *P. fluorescens* and *Saprolegnia parasitica* in rainbow trout lesions and found that *P. fluorescens* could strongly inhibit the growth of the fungus. It was suggested that *P. fluorescens*, or an antibiotic derived from it, might be used in biological control of saprolegniasis.

Snyman et al. (1993) found that *P. fluorescens* genetically engineered to produce the insecticidal toxin from *Bacillus thuringiensis* was toxic to *Eldana saccharina*. An LC_{50} of 1.86 mg freeze-dried bacterial powder/ml of insect diet was calculated, and it successfully reduced sugarcane boring.

P. fluorescens has been shown to have the ability to degrade a wide variety of compounds, including: 3-chlorobenzoic acid (Fava et al., 1993); naphthalene, phenathrene, fluorene and fluoranthene (Weissenfels et al., 1990); chlorinated aliphatic hydrocarbons (Vandenbergh and Kunka, 1988); styrene (Baggi et al., 1983); and pure hydrocarbons and crude oil (Janiyani et al., 1993). *P. fluorescens* can also be used in biosensor applications. For example, the recombinant *P. fluorescens* strain HK9, which lights up in the presence of contaminants such as PAHs (due to the insertion of lux genes), allows easy detection of bioavailable fractions of pollutants in soils and sediments (King et al., 1990).

6.4 *P. fragi*

No information was found on the use of *P. fragi* in environmental applications.

6.5 *P. putida*

P. putida is capable of eliminating phytopathogenic microorganisms and stimulating plant growth (Vancura, 1988; Kloepper et al., 1988; Freitas and Germida, 1990). *P. putida* is also capable of degrading many unusual compounds by means of enzymatic systems encoded in plasmids. Chemicals degraded include polychlorinated biphenyls (PCBs) (Boyle et al., 1993; Lajoie et al., 1994); trichloroethylene (TCE) (Fujita et al., 1995); acetonitrile and sodium cyanide (Babu et al., 1994). *P. putida*

has also shown the ability to remediate non-ionic sewage (Turkovskaya et al., 1993), pulp mill waste (black liquor) (Jain et al., 1993), waste gases using a biofilter (Zilli et al., 1993), electroplating effluent with high concentrations of copper (Cu(II)) (Wong et al., 1993), and high-sulphur coal (Khalid and Aleem, 1991).

6.6 *P. syringae*

Lindow et al. (1988) monitored the fate of a strain of *P. syringae* in experimental field trials in the United States. They found an exponential decrease in numbers of viable cells deposited at increasing distances from sprayed field plots. The relative rate of survival of cells sprayed directly on plants was more than ten times higher than that of cells dispersed through the air to similar adjacent plants.

Use of *P. syringae* has been proposed to enhance snowmaking and to delay frost damage in plants (Lindow, 1983; Wilson and Lindow, 1993b). *P. syringae* has also been shown to incorporate aluminium, chromium and manganese, so the bioremediation of sites contaminated with these chemicals may be a potential use (Alaoukaty et al., 1992).

6.7 *P. tolaasii*

No information was found on the use of *P. tolaasii* in environmental applications.

7. Characterisation of the genome (e.g. presence of large plasmids, insertion sequences) and stability of these characteristics

Members of the genus *Pseudomonas* are known for their metabolic versatility. They are capable of degrading many recalcitrant xenobiotics due to their ability to recruit new genes and alter the expression of existing ones. An understanding of the relative chromosomal position of relevant genes, the diversity of mobile genetic elements found within this genus, and the role these mobile genetic elements play in the stability and metabolic adaptation of individual isolates, can be helpful for regulatory assessments.

The chromosomes of *P. putida* and *P. aeruginosa* have been described in detail by Holloway and Morgan (1986) and by Ratnaningsih et al. (1990), Romling et al. (1989) and Holloway et al. (1994). Holloway et al. (1990a) provide genetic maps of these two species, which are useful in locating the relative positions of important genes and provide a good summary of other chromosomal and extrachromosomal features. The sizes of the chromosomes for *P. putida* and *P. aeruginosa* vary from approximately 4,400 to 5,400 kb, with *P. aeruginosa* strain PAO having a genome size (5,400 kb) significantly larger than the 4,700 kb *E. coli* chromosome. Analysis of the distribution of chromosomal genes in pseudomonads shows that those involved in biosynthesis are not contiguous as with the enterobacteria. The genes for catabolic functions tend to be clustered on the chromosome, but are also not contiguous. Many catabolic functions are located on plasmids (e.g. Table 2); these genes, such as TOL (toluene degradation) and NAH (napthalene degradation), tend to be contiguous. This genome configuration allows for many diverse substrains within a species, each adapted to a particular environment.

Pseudomonas species contain a large variety of plasmids, insertion sequences, and transposons. The diversity of plasmids involved in degradation of organic compounds, drug resistance, and phytopathogenicity is indicated in Tables 2, 4 and 7. Insertion sequences (IS elements) and transposons are mobile within the genome of gram-negative bacteria, and can act as new promoters or as terminators,

causing polar mutations. If two IS elements are located near each other in the appropriate orientation, they can be transposed to a second genome as a unit along with any intervening genes.

These three classes of mobile genetic elements (plasmids, insertion sequences, and transposons) can potentially interact within the same isolate, causing shifts in the positions of key catabolic genes. An example is the NAH plasmid napthalene degradative genes, which are nested within a defective but mobilizable transposon on the plasmid (Tsuda and Iino, 1990). Such shifts can result in a variable stability for some traits. For example, *P. syringae* pv. *savasatoni* mutations causing IAA deficiency were identified to have resulted from the action of two IS elements. In another case, a 150 kb plasmid (able to integrate into the chromosome) from *P. syringae* pv *phaseolicola*, when excised from the chromosome, resulted in the formation of a series of plasmids that either contained chromosomal DNA or were deletion mutants of the plasmid. These events were associated with a common repeated sequence (RS) (Coplin, 1989). In a reverse situation, components of the TOL plasmid have been shown to integrate into both the *P. putida* and *P. aeruginosa* chromosomes (Holloway et al., 1990b), thereby potentially stabilizing degradative genes in the genome of the isolates.

Besides affecting the stability of certain traits, mobile genetic elements allow pseudomonads to recruit new genes from replicons such as plasmids, which can lead to new metabolic capabilities. Specific examples have been given by Chakrabarty (1995) of *P. putida*'s ability to recruit new degradative genes on a transposable element. These new genes allow the organism to degrade new chemicals without the need to evolve completely new degradative pathways. This species has been able to acquire the genes needed to degrade 3-chlorobenzoate to the intermediate protocatechuate, which then is further degraded by resident chromosomal genes. In a similar fashion, the same species has been able to degrade phenol by acquisition of two genes, *pheA* and *pheB*, whose products can convert phenol to intermediates which are metabolized by a chromosomally-encoded *ortho* pathway (Chakrabarty, 1995). A transposon-like mobile element encoding a dehalogenase function has also been recently described in *P. putida* (Thomas et al., 1992). In the well-characterised *P. putida* mt-2 plasmid pWW0, the TOL-degradative enzymes are encoded on a 56 kb transposon which is itself part of a 70 kb transposon (Tsuda et al., 1989), giving rise to a family of TOL plasmids (Assinder and Williams, 1990). In addition to acquisition of degradative genes, pseudomonads can also acquire genes whose products aid in waste degradation.

The chlorosis-inducing phytotoxin coronatine, produced by *P. syringae* pvs. *tomato* and *atropurpurea* is plasmid encoded (Coplin, 1989). Other toxins (e.g. phaseolotoxin, syringomycin and tabtoxin) have been shown to be chromosomally encoded. *P. syringae* pv. *savasatoni* produces abnormal growths due to an imbalance of cytokinin and auxin plant hormones. The genes for their biosynthesis are plasmid encoded in oleander, but not olive pathovars. The majority of *P. solanacearum* strains contain a large (700-1000 kb) megaplasmid that contains genes for host range and pathogenicity.

8. Genetic transfer capability

The ability of pseudomonads to develop new metabolic pathway capabilities is often dependent on an isolate's ability to acquire DNA from other bacteria, which is then integrated into the genome in a manner dependent on the organism's environment. The three common systems for gene transfer in bacteria, namely conjugation, transduction and transformation, have been observed among members of the genus *Pseudomonas.* All three gene transfer mechanisms have been observed under laboratory and natural conditions. Gene transfer by all three mechanisms is affected by biological factors such as the nature and host range of the mobile genetic element, its transfer frequency, the concentrations of recipient and donor organisms, and the presence of other organisms which prey on donors and recipients. Abiotic factors such

as temperature, moisture, and the presence of physical substrates which allow survival and/or gene transfer also affect the transfer frequency.

Even if the DNA is transferred to a new recipient, it may not be expressed. Sayre and Miller (1990) provide a detailed summary of factors associated with transposons and plasmids, the donors and recipients, and other biotic and abiotic conditions which affect gene transfer rates.

Conjugation

The acquisition of genetic material via conjugative plasmids represents an important evolutionary mechanism in the production of strains resistant to antibiotics and heavy metals, and with the ability to mineralise xenobiotics in selective environments. Gene transfer events may even affect the pathology of certain phytopathogens. Changes in cultivar-specificity and a loss of ability to produce fluorescent pigments of *P. syringae* pv. *pisi* were found to result upon the acquisition of IncP1 replicons such as plasmid RP4. Curing the RP4 plasmid from the strain maintained the new phenotype (Moulton et al., 1993). Walter et al. (1987) developed a combined mating technique to measure the conjugal transfer potential of conjugative plasmids that uses four different standard mating techniques (colony cross streak, broth mating, combined spread plate, and membrane filtration), since no one technique worked best for the tested combinations of plasmids and recipients.

Conjugation between pseudomonads has been detected in both soil and aquatic environments. The transfer of conjugative plasmids has been demonstrated to occur between pseudomonads in a number of non-rhizosphere and rhizosphere soil environments, both in microcosms and *in situ* (van Elsas et al., 1988; Trevors and Berg, 1989; Lilley et al., 1994).

Transfer frequencies were found to be enhanced by two orders of magnitude, that is, up to 10^{-2} per recipient organism, on the rhizoplane of sugarbeet *in situ* (Lilley et al., 1994). Soil components (such as clay, silt, organic matter and plant roots) provide excellent surfaces for the cell-to-cell contact required for bacterial conjugation (Trevors and Berg, 1989; Stotzky et al., 1991). In wheat plant root (van Elsas et al., 1988) and sugarbeet (Lilley et al., 1994) conjugation studies, survival of the donor and recipient, as well as frequency of plasmid transfer, decreased with increasing distance from the plant root. Transfer frequencies are also affected by soil moisture, with frequencies for R-plasmid transfer between *E. coli* isolates shown to be optimal at 60 to 80% soil moisture holding capacity (Trevors and Starodub, 1987). Conjugal transfer of broad host range plasmids between *P. aeruginosa* donor and recipient strains in lake water has been observed to occur at a lower rate in the presence of the natural microbial community (O'Morchoe et al., 1988). Plasmids incapable of conjugation themselves have been shown to be mobilised from a laboratory strain of *E. coli* in a laboratory-scale wastewater treatment facility by mobilizer and recipient *E. coli* strains of both laboratory and wastewater origin (Mancini et al., 1987).

The TOL plasmid pWWO can be transferred to other microorganisms, and its catabolic functions for the metabolism of alkylbenzoates are expressed in a limited number of gram-negative bacteria, including members of the rRNA group I pseudomonads and *E. coli* (Ramos-Gonzalez et al., 1991). Transfer of the recombinant plasmid to *Erwinia chrysanthemi* was observed, but transconjugants failed to grow on alkylbenzoates because they lost catabolic functions. Pseudomonads belonging to rRNA groups II, III, and IV, *Acinetobacter calcoaceticus*, and *Alcaligenes* sp. could not act as recipients for TOL, either because the plasmid was not transferred or because it was not stably maintained. Under optimal laboratory conditions, the frequency of transfer of pWWO from *P. putida* as a donor to pseudomonads belonging to rRNA group I was on the order of 1 to 10^{-2} transconjugants per recipient, whereas the frequency of intergeneric transfer ranged from 10^{-3} to 10^{-7} transconjugants per recipient. Intra-species, but not inter-species transfer of TOL in soils has been reported (Ramos et al., 1991), but it was affected by the type of

soil used, the initial inoculum size, and the presence of chemicals that could affect the survival of the donor or recipient bacteria (Ramos-Gonzalez et al., 1991).

The *P. putida* TOL plasmid pWWO and the wide host range RP4 plasmid are able to mediate chromosomal mobilisation in the canonical unidirectional way (i.e. from donor to recipient cells) and bi-directionally [i.e. donor to recipient to donor (retrotransfer)] (Lejeune and Mergeay, 1980; Mergeay et al., 1987; Top et al., 1992; Ramos-Gonzales et al., 1994). Transconjugants are recipient cells that have received DNA from donor cells, whereas retrotransconjugants are donor bacteria that have received DNA from a recipient. The TOL plasmid pWWO and the pRP4 plasmid are able to directly mobilise and retromobilise a chromosomal marker integrated into the chromosome of the other *Pseudomonas* strains, and this process probably involves a single conjugational event. The rate of retrotransfer (as well as direct transfer) of chromosomal markers is influenced by the location of the marker on the chromosome, and it ranges from 10^{-3} to less than 10^{-8} retrotransconjugants per donor (transconjugants per recipient). The mobilised DNA is incorporated into the chromosome of the retrotransconjugants (transconjugants) in a process that seems to occur through recombination of highly homologous flanking regions. No interspecific mobilisation of the chromosomal marker in matings involving *P. putida* and the closely related *P. fluorescens* was observed.

It seems clear that pseudomonads can acquire plasmids from other bacteria in the environment. This premise is supported by the array of plasmids that have been recovered from members of *Pseudomonas,* some of which are listed in Tables 2, 4 and 7. The boundaries to gene transfer events are illustrated by plasmid RP4, originally isolated in *P. aeruginosa,* which has been shown to be transmissible to all gram-negative bacteria tested (Riley, 1989). *E. coli* has been shown to transfer plasmid-borne genetic information to over 40 genera (Stotzky et al., 1991). Direct evidence of pseudomonad isolate acquisition of plasmids from other bacteria in the environment is also available: Bale et al. (1988) showed that an introduced *P. putida* recipient acquired mercury resistance plasmids from an intact lotic epilithic bacterial community at frequencies up to 3.75×10^{-6} per recipient.

Transduction/bacteriophage mediated gene transfer

Two characteristics of a bacteriophage (phage) which are important in determining its ability to broadly distribute DNA were summarised in Sayre and Miller (1990). First, the host range of most phages is restricted to one species or a small number of related taxa, although broad host range phages such as phages P1 and Mu are known. Second, phages which undergo specialised transduction are likely to transfer chromosomal genes which are in close proximity to the phage integration site, while generalised transducing phages can transfer any of the bacterial genome's sequences with approximately equal frequency.

Many different lytic and temperate phages have been identified in *Pseudomonas,* and the morphological diversity among phages is at least as great as for any other bacterial genera. Transduction by temperate phage of *P. aeruginosa* chromosomal DNA has been demonstrated in fresh water microcosms (Morrison et al., 1979; Saye et al., 1987; 1990) and the phylloplane of bean and soy bean plants (Kidambi et al., 1994).

P. aeruginosa has been frequently reported as subject to lysogeny, the process by which the phage chromosome becomes integrated into the bacterial host chromosome and is stably replicated with it, as a prophage. Lysogeny may lead to increased fitness of bacterial strains in the natural environment, by increasing the size and flexibility of the gene pool available to natural populations of bacteria via horizontal gene transfer. Approximately 45% of *Pseudomonas* field isolates tested positive in colony hybridisations when probed with phage isolated from the same area (Miller et al., 1990a). The prophage

appears to contribute a major source of phage in the natural environment. In addition to mediating the transfer of genetic material within and between species, the induction of certain prophages results in transposition and mutagenesis events within the host genome.

Transformation

Both chromosomal and plasmid DNA are subject to natural transformation in the environment, a natural physiological process which is different from the artificial transformation techniques used in the laboratory (Stewart, 1990). In order for transformation of a cell to result in expression of the new DNA sequence, DNA must: 1) be excreted or lost from a donor cell; 2) persist in the environment; 3) be present in sufficient concentrations for efficient transformation to occur; 4) come in contact with a recipient cell which is naturally competent to receive the donor DNA; 5) be able to evade any recipient cell defences which degrade foreign DNA; and 6) integrate into a stably-maintained replicon in the recipient. Marine and soil environments have been shown to contain biologically significant levels of dissolved DNA (Paul et al., 1987; Lorenz et al., 1988). Soil environments offer protection from nuclease digestion for chromosomal and plasmid DNA (otherwise available for transformation) when bound to clay and sediment matter (Lorenz and Wackernagel, 1991; Romanowski et al., 1991; Khanna and Stotzky, 1992).

Natural transformation was found for *P. stutzeri, P. mendocina, P. alcaligenes and P. pseudoalcaligenes,* but not for *P. aeruginosa, P. putida, P. fluorescens,* and *P. syringae strains* (Carlson et al., 1983).

B. Human Health Considerations

9. Diseases caused and mechanism of pathogenicity including invasiveness and virulence

9.0 General considerations

Included amongst the rRNA group I fluorescent pseudomonads are species pathogenic to humans. Those that do cause infection are generally opportunists, promoting a variety of conditions ranging from endocarditis to dermatitis (Pollack, 1992; Artenstein and Cross, 1993; Berger et al. 1995; Jarvis and Skipper, 1994). Individuals most at risk from *Pseudomonas* infection are the immunocompromised, in particular individuals with AIDS, patients with cystic fibrosis, and those suffering major trauma or burns (Artenstein and Cross, 1993; Neu, 1985; Bodey et al., 1983; Moayyedi et al., 1994; Schuster and Norris, 1994).

It should be stressed that only some of the Pseudomonads have been implicated in human disease and that proponents should not base assessment criteria on *P. aeruginosa,* the most problematic member of the genus. Proponents should also be aware that not all strains of a given species have been shown to promote disease, and that assessment criteria should be based upon the strain being reported rather than the species as a whole.

9.1 *P. aeruginosa*

The predominant pseudomonad isolated from clinical sources is *P. aeruginosa*. Evaluation of the pathogens causing nosocomial infections in hospitals in the United States (Jarvis and Martone, 1992) indicated that *P. aeruginosa* was the fourth most common pathogen isolated (10.1%) in hospital-wide surveillance and the most common (12.4%) in intensive care units. In more recent surveys, *P. aeruginosa* was found to be the fifth most common pathogen (9%) (Emori and Gaynes, 1993) and increased to 29% in intensive care facilities [European Prevalence of Infection in Intensive Care Study (EPIC) (Spencer, 1994)]. The ability of *P. aeruginosa* to persist in a number of hospital disinfectants and pharmaceuticals (Dominik et al., 1995; Gilardi, 1991) and in sanitary facilities (Bobhammer et al., 1996; Döring et al., 1991; Zembrzuska-Sadkowska et al., 1995) probably contributes to its prevalence in the hospital environment. *P. aeruginosa* causes a wide range of syndromes, involving nearly all body systems, that vary from sub-acute to chronic (Artenstein and Cross, 1993; Pollack, 1992).

Bacteremia associated with *P. aeruginosa* is restricted mainly to immunocompromised individuals with significant underlying disease (Artenstein and Cross, 1993; Askamit, 1993; Spencer, 1994). Symptoms are indistinguishable from gram-negative sepsis caused by other bacteria (Pollack, 1993). Factors associated with bacteremia include malignancy, transplants (solid organ, bone marrow), diabetes, cirrhosis of the liver, renal failure, burns, trauma, intravenous drug abuse, corticosteroid therapy, cytotoxic chemotherapy, cardiopulmonary resuscitation, immunoglobulin deficiency, AIDS, broad spectrum antibiotics, and invasive instrumentation (IV catheters, endoscopes, mechanical ventilation, etc.) (Askamit, 1993; Artenstein and Cross, 1993; Dropulic et al., 1995; Mallolas et al., 1990; Nensey et al., 1990). Major entry portals for *P. aeruginosa* bacteremia appear to be following infection (or colonisation) of the respiratory tract (Gallagher and Watanakunakorn, 1990; Artenstein and Cross, 1993) and the genitourinary tract (Gallagher and Watanakunakorn, 1990; Aksamit, 1993).

. *P. aeruginosa* septicemia is primarily a condition of debilitated, immunocompromised adults and of infants. It is usually acquired in hospital, with prior broad-spectrum antibiotic therapy as a predisposing factor (Bodey et al., 1983; Richet et al., 1989), although community-acquired infections in apparently healthy children (Ros, 1989) and adults (Ishihara et al., 1995) have been reported. Mortality is high in immunocompromised patients (up to 50%) (Artenstein and Cross, 1993; Bisbe et al., 1988), with a better prognosis in “normal healthy” individuals (Ishihara et al., 1995).

Endocarditis due to *P. aeruginosa* has been associated with two major predisposing factors (Artenstein and Cross, 1993): the use of prosthetic heart valves and the use of illicit parenteral drugs. In drug abusers, endocarditis usually occurs in the right side of the heart and is sub-acute, although a complication, septic pulmonary emboli involving the tricuspid valve, can occur (Pollack, 1992).

Nosocomial pneumonia is the second most common hospital-acquired infection in the United States (Aksamit, 1993; Emori and Gaynes, 1993). *P. aeruginosa* is the most prevalent etiological agent for both poly- and mono-microbial pneumonia (Emori and Gaynes, 1993). Community-acquired pneumonia has also been attributed to this organism; its occurrence is rare (Artenstein and Cross, 1993), but it is more frequently recognised in AIDS patients (Dropulic et al., 1995; Schuster and Norris, 1994). Colonisation of the oropharyngeal and/or the upper gastrointestinal tracts is an important precursor to nosocomial pneumonia, although colonisation does not always imply infection. However, susceptibility to pneumonia is inversely related to a patient’s basic health (Aksamit, 1993; Artenstein and Cross, 1993, Dick et al., 1988; Dropulic et al., 1995). Conditions predisposing to *P. aeruginosa* pneumonia are similar to those mentioned for bacteremia.

Bacteremic pneumonia, with organisms isolated from both the lung and bloodstream, resembles bacteremia and pneumonia in clinical presentation. Prognosis is bleak with this syndrome. Mortality rates of 80-100% are observed, compared to 27-50% for bacteremia and 30-60% for pneumonia (Aksamit, 1993).

Chronic pulmonary colonisation by *P. aeruginosa* in the lungs of patients with cystic fibrosis results in frequent acute episodes of pneumonia and chronic bronchiectasis, but rarely in bacteremic pneumonia (Aksamit, 1993). Chronic infection leads to the obstruction of the airways, respiratory distress, and eventually death (Gilligan, 1991; Romling et al., 1994).

Otolaryngologic infections due to *P. aeruginosa* range from superficial and self-limiting to life-threatening (Artenstein and Cross, 1993). The most serious ear infection due to this organism is malignant otitis externa, usually resulting from a failure of topical therapy, and resulting in an invasive disease-destroying tissue which may progress to osteomyelitis at the base of the skull and possible cranial nerve abnormalities (Artenstein and Cross, 1993). Other ear infections associated with *P. aeruginosa* include external otitis (swimmer's ear), otitis media, chronic suppurative otitis media, and mastoiditis (Artenstein and Cross, 1993; Legent et al., 1994; Kenna, 1994; Pollack, 1992).

P. aeruginosa is the leading cause of gram-negative ocular infections, presenting as keratitis or endophthalmitis (Holland et al., 1993; Chatterjee et al., 1995; Bukanov et al., 1994). Predisposing factors include the use of contact lenses (in particular their cleaning and storage solutions), trauma, burns, ocular irradiation, compromised host defences, and systemic infections (Holland et al., 1993; Pollack, 1992; Imayasu et al., 1994; Stapleton et al., 1995).

Moisture is the paramount defining factor in *P. aeruginosa* growth. Normal dry skin does not support growth, whereas moist skin enables the organism to flourish. For this reason, dermatologic infections with *P. aeruginosa* tend to be more prevalent in moist tropical and subtropical climates (Bodey et al., 1983) or to be associated with the use of swimming pools, hot tubs or whirlpools (Gustafson et al., 1983; Trueb et al., 1994; Vesaluoma et al., 1995). The use of contaminated "loofah" cosmetic sponges is another source of *P. aeruginosa* infection (Bottone and Perez, 1993; 1994; Fisher, 1994). Folliculitis, pyoderma, cellulitis and ecthyma gangrenosum are all dermatologic infections in which *P. aeruginosa* has been implicated (Pollack, 1992; Artenstein and Cross, 1993; Gustafson et al., 1983; Fisher, 1994; Noble. 1993).

P. aeruginosa is a frequent isolate from wounds, particularly those contaminated with soil, plant material or water. Its presence may reflect colonisation as opposed to infection, which is a consequence of its ubiquitous distribution in nature (Artenstein and Cross, 1993; Pollack, 1992). Puncture wounds, particularly those penetrating to bone, may result in osteomyelitis or osteochondritis. The former is common in intravenous drug abusers (Artenstein and Cross, 1993) and the latter in puncture wounds to the foot in children and diabetics (Lavery et al., 1994; Pollack, 1992; Jarvis and Skipper, 1994). The wearing of tennis shoes (sneakers) at the time of puncture injury increases the chance of *P. aeruginosa* infection (Pollack, 1992; Lavery et al., 1994; Fisher et al., 1985).

In rare cases, *P. aeruginosa* has been associated with meningitis or brain abscess (Pollack, 1992) and infection of the gastro-intestinal tract (Artestein and Cross, 1993). Both conditions are nosocomially acquired, occurring in patients suffering from malignancies, invasive procedures or neutropenia (Pollack, 1992; Artenstein and Cross, 1993).

9.2 *P. fluorescens*

P. fluorescens has occasionally been associated with human infection. The inability of most strains to grow at normal human body temperature (Palleroni, 1992a) restricts invasion and subsequent disease promotion. This organism has the ability to grow at 4°C (Gilardi, 1991). This characteristic, along with the observation that it is isolated from the skin of a small percentage of blood donors, makes it an occasional contaminant of whole blood and blood products (Puckett et al., 1992; Stenhouse and Milner, 1992). Pseudobacteremia may result from the infusion of contaminated products (Scott et al., 1988; Simor et al., 1985; Gottlieb et al., 1991; Foreman et al., 1991) or from the use of contaminated equipment (Anderson and Davey, 1994).

P. fluorescens has been occasionally isolated from patients with AIDS (Franzetti et al., 1992; Roilides et al., 1992), where it caused bacteremia and urinary tract, ocular and soft tissue infections. Chamberland et al. (1992), in their across-Canada survey of septicemia, found that 1.5% of isolates were *P. fluorescens*. It is apparent that *P. fluorescens* can be an opportunistic pathogen in cancer patients and in others who are severely immunocompromised, but that it is of little concern to immunocompetent individuals. *P. fluorescens* is occasionally found in sputa of patients with cystic fibrosis, although its role as a pathogenic factor has yet to be resolved.

9.3 *P. fragi*

P. fragi is one of the pseudomonads associated with food spoilage (Barrett et al., 1986; Drosinos and Board, 1995; Greer, 1989) and is commonly isolated from milk products, pork and lamb. A search of the literature dating back to 1966 failed to reveal any association between *P. fragi* and human disease.

9.4 *P. putida*

P. putida is a rare opportunistic pathogen in immunocompromised individuals. Like *P. fluorescens*, this organism can grow at 4°C in whole blood and blood products and is consequently an occasional source of pseudobacteremia (Pitt, 1990; Taylor et al., 1984; Tabor and Gerety, 1984). Septicaemia and septic arthritis due to *P. putida* in immunocompromised patients have been reported (MacFarlane et al., 1991; Madhavan et al., 1973) and bacteremia in AIDS patients can occur at low frequency (Roilides et al., 1992). All syndromes appear to be associated with breaching of the patient's mechanical defences, either associated with transfusion or following placement of in-dwelling catheters.

9.5 *P. chlororaphis, P. syringae, P. tolaasii*

A search of the literature dating back to 1966 failed to reveal any association between these species and human disease. The possibility does exist that an incomplete identification has failed to speciate these organisms, and that they are reported in the literature as *Pseudomonas* sp.

10. Communicability

P. aeruginosa, the species of most concern in the rRNA group I pseudomonads, has a ubiquitous distribution at a low frequency in nature (Romling et al., 1994a). Outside of the hospital environment, 20 to 30% of people harbour faecal *P. aeruginosa*. This frequency increases during hospitalisation as a result of contact with an environment in which the organism is more common. Both healthy individuals and patients with P. aeruginosa infections may serve as reservoirs for infection in hospitals.

P. aeruginosa is an important cause of nosocomial infections. It is particularly a problem in burn units, neonatal units, and wards housing leukemia and other cancer patients (Bergen, 1981). Nosocomial infections may spread by transmission 1) directly between patients; 2) via medical personnel; 3) via inanimate objects which may serve as reservoirs or vectors; and 4) from the normal flora of the patient (i.e. autoinfection).

Most types of hospital equipment or utensils can serve as a source of infection, including pharmaceutical products, disinfectants, water jugs, table tops, trays, urine bottles, urethral catheters, anaesthetic equipment, and respiratory apparatus. Transmission may also occur via food stuffs such as strawberries, plums and other fruit, vegetables, frozen poultry, refrigerated eggs, lemonade, raw milk, and any equipment or utensil involved in the preparation or serving the food.

11. Infective dose

Infective dose for the fluorescent pseudomonads is not really relevant, since infection usually occurs in immunosuppressed individuals. Most patients suffering from cystic fibrosis acquire a *P. aeruginosa* infection at some stage of their lives, resulting in frequent, recurrent bouts of pneumonia. Mortality in such cases may reach 100%.

12. Host range, possibility of alteration

P. aeruginosa has a broad host range which includes humans, animals, and some plants. It converts from a non-mucoid state to a mucoid, alinate-producing variant in the lungs of CF patients. The mucoid form is almost exclusive to colonisation of this site. Upon *in vitro* propagation, the mucoid strains isolated from CF lungs may undergo a spontaneous reversion to the non-mucoid form (Maharaj et al., 1992).

13. Capacity for colonisation

Fluorescent pseudomonads may be found in the normal bacterial flora of the intestines, mouth or skin of humans or animals. Colonisation is harmless under normal circumstances. In immunosuppressed or immunocompromised patients the capacity for colonisation by *P. aeruginosa* is high.

14. Possibility of survival outside the human host

rRNA group I fluorescent pseudomonads do not require human or animal hosts for survival. Most are common residents of soil, rhizosphere, sediment, and aquatic habitats. These generally moist environments provide natural reservoirs for the organisms. The pseudomonads have modest nutritional demands and can survive for months in tap water, distilled water, sink drains, or any other moist environment.

15. Means of dissemination

The fluorescent pseudomonads are ubiquitous microorganisms. Anyone (not only infected individuals), or anything, may serve as a source or vector for dissemination (refer also to 10 and 26).

16. Biological stability

In *P. aeruginosa* infections of the CF lung, a transition from a non-mucoid to a mucoid, alginate producing variant is observed, indicating the pleomorphic nature of this organism. Furthermore, the level of toxin production varies with the isolate, suggesting that expression levels of chromosomally encoded genes are subject to strain differences. Recent studies indicate that this variation is attributable to the variable position of the genes on the chromosome, due at least in part to chromosome reassortment and the movement of IS-like sequences (Vasil et al., 1990).

17. Antibiotic-resistance patterns

17.1 *P. aeruginosa*

P. aeruginosa is naturally resistant to many widely used antibiotics. Resistance in part is thought to be the result of an impermeable outer membrane and the production of extracellular polysaccharides (Quinn, 1992). The organism is usually resistant to low levels of kanamycin, penicillins (with the exception of the anti-pseudomonal penicillins: carbenicillin, ticarcillin, piperacillin), most of the first and second generation cephalosporins, chloramphenicol, nalidixic acid, tetracyclines, erythromycin, vancomycin, sulfonamide, trimethoprim and clindamycin (Wiedemann and Atkinson, 1991). Antibiotic resistance is often due to the presence of plasmids (Table 4). Individual strains may be resistant to antibiotics to which the species is generally susceptible. For this reason, antibiotic resistance patterns should not be relied on for species verification, but should be assessed on a case-by-case basis.

17.2 *P. fluorescens* and *P. putida*

Antibiotic resistance patterns for *P. fluorescens* and *P. putida* are difficult to assess, since only small numbers of isolates have been tested in controlled studies. The organisms tested are susceptible to low levels of kanamycin and resistant to carbenicillin and gentimicin, two of the antibiotics still in use against *P. aeruginosa* (Pitt, 1990). Again the use of antibiotic resistance/susceptibility profiles should be regarded with caution, since variation within a species may be great.

17.3 *P. chlororaphis*, *P. fragi*, *P. syringae*, *P. tolaasii*

Antibiotic susceptibility patterns for these species were not found in the literature searched.

18. Toxigenicity

The pathogenicity of *P. aeruginosa* is accredited to the wide array of virulence-associated factors produced by some if not all strains. Pili act as adhesins to a variety of cell types and enable the organism to colonise epithelial surfaces (Prince, 1992). Once established, the bacteria secrete a number of extracellular products capable of tissue damage and facilitating dissemination of the bacteria (Plotkowski

et al., 1994). Proteases (including elastase), exotoxin-A, exoenzyme-S, phospholipase-C, exolipase, rhamnolipid, alginate, cytotoxin, high molecular weight leukocidin and endotoxin have all been implicated in pathogenesis (Fick, 1993; Govan and Nelson, 1992; Holder, 1993; Holland et al., 1993; Jaeger et al., 1991; McCubbin and Fick, 1993; Kudoh et al., 1994; Lutz et al., 1991; Noda et al., 1991).

Exotoxin-A (ETA) and exoenzyme-S are ADP-ribosyltransferases which inhibit protein synthesis in the eukaryotic cell. ETA is produced during the decline of the *P. aeruginosa* growth cycle. Its synthesis is dependent on the iron concentration in the growth medium (Stephen and Pietrowski, 1986). The levels of both ETA and exoenzyme-S vary with the isolate examined.

Phospholipase-C (PLC) is another extracellular enzyme produced by *P. aeruginosa* which is toxic in micro or sub-microgram levels. PLC preferentially degrades phospholipids, which are plentiful in the eukaryote cell. In addition, one of the substrate products of lipid degradation by PLC (diacylglycerol) can have toxic effects on the host animal by inducing the production of potent substances (arachronic acid metabolites and protein kinase C). These by-products alter eukaryotic cell metabolism and incite inflammatory responses.

Elastase, one of the extracellular proteases, degrades elastin, collagen, human immunoglobulin and serum α-1-proteinase inhibitor (Iglewski et al., 1990), activities which help evade the immune response and sponsor tissue invasion. Alkaline protease, another of the extracellular proteases, is active on IgA, cytokines (TNF-α; IFN-γ; IL-2), lactferrin and transferrin, fibrinogen, and fibrin (Shibuya et al., 1991; Doring et al., 1988; Frick et al., 1985; Parmely et al., 1990). These enzymatic activities promote disruption of respiratory cilia and increased vascular permeability, which probably contribute to establishment in the lung and resulting pneumonia.

The toxigenic potential of other species of *Pseudomonas* is less well studied. Proteases and phospholipases have been detected in some strains of *P. fluorescens* and *P. putida*, but their significance in human infection has yet to be elucidated.

19. Allergenicity

Fluorescent pseudomonads have not been described as potent allergens. However, they do possess endotoxin (lipopolysaccharide), which may precipitate an allergic response in some individuals.

Table 4

Examples of plasmids encoding for drug resistances in *P. aeruginosa*

Plasmid	Resistances encoded
RP1	carbenicillin, kanamycin, neomycin, tetracycline
RP1-1	carbenicillin
R9169	carbenicillin, kanamycin, neomycin, tetracycline
R6886	carbenicillin, kanamycin, neomycin, tetracycline
RP8	carbenicillin, kanamycin, neomycin, tetracycline
R2-72	carbenicillin, streptomycin, kanamycin
R38-72	tetracycline, streptomycin
R39-72	tetracycline, streptomycin
R931	tetracycline, streptomycin
R679	streptomycin, sulphonamide
R1162	streptomycin, sulphonamide
R3108	streptomycin, sulphonamide, tetracycline
R209	streptomycin, sulphonamide, gentamicin
R130	streptomycin, sulphonamide, gentamicin
R716	streptomycin
R503	streptomycin
R5265	streptomycin, sulphonamide
R64	ampicillin, carbenicillin, sulphonamide, gentimicin, kanamycin
R40a	ampicillin, anamycin, paromycinin, sulphonamide

taken from Trevors (1991)

20. Availability of appropriate prophylaxis and therapies

20.1 *P. aeruginosa*

Antibiotic therapy for *P. aeruginosa* depends upon the site of infection and the relative susceptibility of the particular strain to the antibiotics tested. Generally, the species is susceptible to very few antibiotics. Ceftazidime, cefsulodin, imipenem, ticarcillin-clavulanic acid, azlocillin, piperacillin, the aminoglycosides, colistin and ciprofloxacin are some of the antibiotics with a high percentage of susceptible isolates (Chamberland et al., 1992; Wiedemann and Atkinson, 1991; Legent et al., 1994). Combination therapy using two effective antibiotics may increase the clinical cure rate in some infections (Lucht et al., 1994), and synergistic combinations of an aminoglycoside with a β-lactam (that has activity against *Pseudomonas*) have continued to be effective (Sepkowitz et al., 1994). The particular antibiotic regime selected will depend, however, on the strain in question and cannot be answered in a generic manner.

20.2 *P. fluorescens* and *P. putida*

Ceftazidime (Jones et al., 1989; Watanabe et al., 1988), imipenem (Jones et al., 1989) and meropenem (Jones et al., 1989) have been described as active against *P. fluorescens*. Antibiotics active against *P. putida* are ceftazidime, carbapenems, aminoglycosides, tetracyclines and polymixin B (Kropec et al., 1994; Bergen, 1981; Papapetropoulou et al., 1994). Any possible treatment regime should be proposed for the strain in question and not based on generic information for the species.

20.3 *P. chlororaphis, P. fragi, P. syringae, P. tolaasii*

No antibiotic susceptibility data for these species were found in the literature searched.

C. Environmental and Agricultural Considerations

21. Natural habitat and geographic distribution. Climatic characteristics of original habitats

21.0 General considerations

. *Pseudomonas* species have been isolated from a wide variety of habitats including soils, fresh or sea water, clinical specimens and laboratory reagents (including distilled water), food stuffs and wastes, flowers, fruit, vegetables, and diseased and healthy plants and animals. Many species appear to have a global distribution. For example, beneficial colonizers and *Pseudomonas*-incited plant diseases (such as *P. syringae*) are worldwide in distribution and involve representatives of most major groups of common plants.

Although pseudomonads are often considered to be ubiquitous, there are also many reports of niche specialisation. For instance, the number of epiphytic bacteria (such as *P. syringae*) present on the leaves of newly emerged plants is very low, indicating that the soil does not appear to serve as an important source of inoculum (Lindow, 1992) or habitat. As well, many phytopathogenic pseudomonads

can only be isolated from the diseased host. For example, *P. syringae* isolates are generally only found in association with live plants or propagative material, and in these niches they appear as virtually homogeneous populations (Schroth et al., 1981). At present, the distribution of these pathogens in the absence of the host is unclear.

21.1 *P. aeruginosa*

. *P. aeruginosa* is widely distributed in soil (Bradbury, 1986) and water (Palleroni, 1984). It is occasionally isolated from both healthy and diseased plants (Bradbury, 1986). Experiments conducted with lettuce and bean under varying conditions of temperature and humidity indicated that *P. aeruginosa* can colonise these plants under conditions of high temperature and humidity (27ºC, 80-95% humidity) (Green et al., 1974). The occurrence declined in lettuce and bean when the temperature and humidity were lowered (16ºC, 55-75% humidity).

Cho et al. (1975) studied the occurrence of *P. aeruginosa* on the foliage and in the soil of potted ornamental plants in order to determine their importance as a disseminating agent in hospital environments. They concluded that although potted plants are potential carriers for introduction of the species to hospital environments, there is no evidence that these plants constitute a primary source of bacteria for hospital infections. Results of a study to determine the prevalence of bacteria in passerines and woodpeckers suggest that *Pseudomonas* spp., including *P. aeruginosa*, are not uncommon in the gut flora of omnivorous and granivorous birds (Brittingham et al., 1988).

21.2 *P. chlororaphis*

P. aureofaciens (*P. chlororaphis*) was one of the most commonly occurring bacteria in soil, and on roots and leaves of both sugarbeet and spring wheat, during the growing season (De Leij et al., 1994). *P. chlororaphis* has also been isolated from water and from dead larvae of cockchafer, a large European beetle (Palleroni, 1984).

21.3 *P. fluorescens*

P. fluorescens is commonly found on plant surfaces, as well as in decaying vegetation, soil and water (Bradbury, 1986). It can be isolated from soil, water, plants, animals, the hospital environment, and human clinical specimens. It is commonly associated with spoilage of foodstuffs such as fish and meat (Gilardi, 1991). The presence of *P. fluorescens* in the rhizosphere of plants has been widely reported. For example, Milus and Rothrock (1993) found *P. fluorescens* to be a very good coloniser of wheat roots, and Lambert et al. (1990) found *P. fluorescens* to be one of the most frequently occurring bacteria on root surfaces in young sugar beet plants in Belgium and Spain.

P. marginalis (*P. fluorescens*) is ubiquitous in soil and is often an internal resident of plant tissues (Schroth et al., 1992). Cuppels and Kelman (1980) detected *P. marginalis* in a Wisconsin river and lake, field soils, root zones of potato plants, washwater from a potato chip processing plant, and decaying carrot and cabbage heads. Strains were found in Wisconsin soils just after the spring thaw, and thus probably overwintered there.

21.4 *P. fragi*

P. fragi has been found associated with refrigerated meat and dairy products (Jay, 1992).

21.5 *P. putida*

P. putida is very common in soils and plant rhizospheres (Palleroni, 1984). Gilardi (1991) indicated the species can be isolated from soil, water, plants, animal sources, the hospital environment, and human clinical specimens. It can be isolated from soil and water after enrichment in mineral media with various carbon sources.

P. putida appears to have a broad global distribution. Sisinthy et al. (1989) isolated the species from soil samples collected in, and around, a lake in Antarctica. However, particular strains may have a more restricted distribution. Chanway and Holl (1993) studied strains obtained from spruce seedling rhizospheres at two different locations in British Columbia, Canada, and found two distinct strains based upon analysis of fatty acids. When the origin of the spruce seed was matched with that of the inoculated *P. putida* strain, a significant increase in the amount and rate of seedling emergence was detected compared to unmatched tests of seedling emergence, suggesting ecotype specificity of strains.

21.6 *P. syringae*

P. syringae occurs naturally among the microflora that inhabit the leaf surface of plants that are typically found in temperate and Mediterranean climates (Wilson and Lindow, 1994; Bradbury, 1986). *P. syringae* survives in association with the host plant and propagative material from the host plant. There is little evidence to suggest that these bacteria survive in soil. They may, however, survive in soil in association with residues of diseased plants, having some capacity to colonise root systems (both host and non-host plant). Stone or pome fruit pathogens, such as *P. syringae*, exist in lesions, cankers or tumours. Inoculum is therefore available for dissemination under favourable environmental conditions. Most of the *P. syringae* group appears to have the capacity to survive as epiphytes on protected parts of healthy leaves, in the buds of the host, and even on non-host plants.

21.7 *P. tolaasii*

P. tolaasii is a natural inhabitant of peat and lime used for casing material in the production of commercial mushrooms, and can be easily isolated from compost after pasteurisation (Howard et al., 1994). In the commercial production of mushrooms, high relative humidity and surface wetness encourage the expression of symptoms of brown blotch caused by *P. tolaasii* (Howard et al., 1994). Symptoms of brown blotch occur more frequently on mushrooms that remain wet for a long time, and in places where they touch one another (Howard et al., 1994). Brown blotch, the mushroom disease caused by *P. tolaasii*, has been reported on all continents except Africa (Bradbury, 1986; Suyama and Fujii, 1993).

22. Significant involvement in environmental processes, including biogeochemical cycles and potential for production of toxic metabolites

Pseudomonads can have a significant involvement in a variety of environmental processes, including important biogeochemical cycles. For example, certain *Pseudomonas* species have the capacity for denitrification producing dinitrogen gas from nitrate. These species include *P. aeruginosa*, *P. fluorescens* (biotypes I and III) and *P. chlororaphis* (Palleroni, 1984).

Toxic metabolites of hazardous wastes

The microbial degradation of a hazardous waste may result in mineralization of the parent waste, or in partial degradation of the parent waste to products which may be toxic. Some microorganisms may

not initially produce problematic metabolites. However, loss of a lower portion of a degradative pathway due to genetic instability may result in the generation of toxic metabolites. These toxic metabolites may result in death of the cell, thus limiting the metabolite's production. The metabolite may also be released from the cell to soil or water and become rapidly inactivated or mineralised by other physical or biological processes. On the other hand, some metabolites may be released from the cell, remain stable in the environment, and have toxic effects equivalent to, or greater than, the parent hazardous waste. There is also a possibility that a microorganism will not produce a metabolite of concern when presented with a single waste, but will produce toxic metabolites in the presence of a complex mixture of related compounds.

Many examples of hazardous waste metabolites have been detected in laboratory experiments, but no well-documented field studies on metabolite formation have been conducted. In many instances, the metabolites produced by one organism will be degraded further or mineralised by others in the immediate environment. For example, TCE epoxide and phosgene are likely degradation products from methanotrophic degradation of trichloroethylene and chloroform, respectively (Alvarez-Cohen and McCarty, 1991). Although these compounds are toxic in mammalian systems, both are also highly reactive and would likely react intracellularly and/or not persist in the environment once released from the cells. Examples of the potential for hazardous metabolites include the production and accumulation of formamide from cyanide as a result of cyanide degradation by *P. fluorescens* strain NCIMB 11764 (Kunz, et al., 1992). As well, Castro and Belser (1990) demonstrated that *P. putida* PpG-786 can dehalogenate 1,1,2-trichloroethane by two pathways under aerobic conditions. The dominant pathway is oxidative and leads to chloroacetic acid and glyoxylic acid. However, a competitive reductive pathway occurs simultaneously and yields vinyl chloride exclusively.

Complex mixtures can result in dead-end metabolite production, or failure to degrade one of the parent compounds, even though the individual wastes can be mineralised individually. Benzene, toluene, and *p*-xylene (BTX) are common contaminants of drinking water, and each individual BTX compound can be mineralised by naturally occurring organisms. However, a combination of the three cannot be mineralised naturally, and can result in accumulation of 3,6-dimethylcatechol from *p*-xylene and a lack of degradation of benzene (Lee et al., 1995).

23. Pathogenicity – host range, infectivity, toxigenicity, virulence, vectors

23.0 General considerations

The fluorescent rRNA group I pseudomonads exhibit a range of pathogenicity characteristics. Some species have not been implicated in animal or plant disease. Other species may be opportunistic pathogens for weakened individuals. The fluorescent rRNA group I pseudomonads also include plant pathogens.

23.1 *P. aeruginosa*

Pathogenicity to animals

P. aeruginosa may be found as part of the normal bacterial flora of the intestines, mouth or skin of animals (e.g. cattle, dogs, horses, pigs). It has a broad host range among animals, which may also extend to plants. Under normal circumstances, colonisation is harmless and infection only occurs when

local or general defence mechanisms are reduced. *P. aeruginosa* is usually associated with disease in individuals with low resistance to infection.

In susceptible hosts *P. aeruginosa* may cause infection at any site, particularly wounds and the respiratory tract. It can cause endocarditis, meningitis, pneumonia, otitis, vaginitis and conjunctivitis. Host defence mechanisms against *P. aeruginosa* are very low in mink and chinchilla, in which the bacterium can spread rapidly, causing fatal disease (Bergen, 1981).

P. aeruginosa has been associated with disease in pigs, sheep and horses (Hungerford, 1990), as well as cattle (Hamdy et al., 1974). Sheep inoculated epicutaneously with *P. aeruginosa*, and then wetted, can rapidly develop a bacterial exudative dermatitis (Hungerford, 1990). *P. aeruginosa* has been reported as the etiological agent in outbreaks of acute infectious disease in mink (Wang, 1987) and was the suspected etiological agent in a report of fatal bronchopneumonia and dermatitis in an Atlantic bottle-nosed dolphin (Diamond and Cadwell, 1979). It has also been reported to be associated with pathogenicity in ducks (Safwat et al., 1986), turkeys (Hafez et al., 1987), Japanese ptarmigan (Sato et al., 1986), and pheasant chicks (Honich, 1972) and to be the causal agent of a disease in broiler fowl in several countries.

P. aeruginosa was reported as one of the causative agents of infectious stomatitis or "mouthrot" in snakes (Draper et al., 1981), although it has been suggested that it is an opportunistic invader rather than an exogenous pathogen in snakes (Draper et al., 1981; Jacobson et al., 1981). Frogs (*Rana pipiens*) that were intraperitoneally injected with high doses (10^4-10^6 bacteria) of *P. aeruginosa* showed significant mortality under stressful conditions (Brodkin et al., 1992). *P. aeruginosa* has been associated with pathogenicity in Nile fish (Youssef et al., 1990) and catfish (*Clarias batrachus*) (Manohar et al., 1976) and as the etiological agent of fin rot in *Rhamdia sapo* (Angelini and Seigneur, 1988).

P. aeruginosa has been associated with pathogenicity in the tobacco hornworm (*Manduca sexta*) (Horohov and Dunn, 1984) and seven species of Lepidoptera, including the silkworms *Pericallia ricini* and *Bombyx mori* (Som et al., 1980). Experimental inoculation of honeybees, by dipping in a bacterial suspension of *P. aeruginosa,* resulted in a 70% death rate within 50 hours (Papadopoulou-Karabela et al., 1992). Dorn (1976) reported *P. aeruginosa* to be responsible for disease outbreaks in laboratory populations of the milkweed bug *Oncopeltus fasciatus*.

The abundant extracellular products of *P. aeruginosa* are thought to contribute to its adverse effects. These products include toxin A, alkaline protease, alkaline phosphatase, lipase, phospholipases and elastase. Toxin A is toxic to animals, with a mean lethal dose in mice of about 0.2 μg when injected intraperitoneally or 0.06 μg when injected intravenously (Nicas and Iglewski, 1986). Toxin A is produced by about 90% of clinical isolates, and a chromosomal location has been established for the structural gene (Nicas and Iglewski, 1986). Most strains produce several extracellular proteases. For mice injected intravenously, the LD_{50} of the alkaline protease and the elastase is 375 and 300 μg respectively (Nicas and Iglewski, 1986). *P. aeruginosa* proteases are reported to be toxic to insects (*Galleria mellonella)* (Lysenko, 1974). *P. aeruginosa* also produces the haemolytic extracellular product phospholipase C, which causes hepatic necrosis and pulmonary edema when injected interperitoneally, and rhamnolipid, which has an LD_{50} of 5 mg when injected interperitoneally into mice (Nicas and Iglewski, 1986).

Pathogenicity to plants

P. aeruginosa has been described as an opportunistic invader of plants (Bradbury, 1986). It has been reported to cause blight disease in bean plants (El Said et al., 1982), and to have caused a lethal palm blight (Bradbury, 1986). Slow soft rot has been produced in plant tissue upon inoculation with strains of *P. aeruginosa* isolated from both animals and plants, and lesions and some necrosis have been found in

tobacco leaves when inoculated with the bacterium (Bradbury, 1986). In a study involving 46 strains of *P. aeruginosa* isolated from human, plant and soil sources, the ratio of pathogenic to non-pathogenic strains for vegetables was 5:1 (Lebeda et al., 1984).

More recently, two strains of *P. aeruginosa* (a clinical isolate and a plant isolate) have been found to elicit severe soft rot symptoms in the leaves of inoculated *Arabidopsis thaliana* plants from certain ecotypes but not others (Rahme et al., 1995). These authors suggested that a strain that exhibited ecotype specificity would most likely be a true plant pathogen, in contrast to a strain that has no capacity to be a plant pathogen under natural settings but infects plants as a consequence of the artificial environment of a laboratory. The same two strains of *P. aeruginosa* were found to cause significant mortality in a mouse burn model. The authors identified genes encoding three virulence factors (*toxA, plcS* and *gacA*) that were required for the full expression of pathogenicity in both plants and animals.

23.2 *P. chlororaphis*

Pathogenicity to animals

A strain of *P. chlororaphis* has been reported to cause disease in salmon fry (*Oncorhynchus rhodurus*) and to kill trout, carp and eel, when inoculated (Egusa, 1992). This strain was judged to be pathogenic to fish (Hatai et al., 1975). *P. chlororaphis* has also been reported to inhibit egg hatch of the nematode, *Criconemella xenoplax*, at a concentration of $2x10^8$ cfu/ml (Westcott and Kluepfel, 1993). Shahata et al. (1988) reported that *P. chlororaphis* infected chickens.

Pathogenicity to plants

P. chlororaphis has been reported as the causal agent for a disease in straw mushrooms (*Volvariella volvacea*) in Puerto Rico, characterised by basal soft rot, internal water-soaking and discoloration (Hepperly and Ramos-Davila, 1986).

23.3 *P. fluorescens*

Pathogenicity to animals

P. fluorescens can infect a wide range of animals including horses (Sarasola et al., 1992), chickens (Lin et al., 1993), marine turtles (Glazebrook and Campbell, 1990), and many fish and invertebrate species. However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

P. fluorescens is considered a secondary invader of damaged fish tissues, but may also be a primary pathogen (Roberts and Horne, 1978; Stoskopf, 1993). The species causes bacterial tail rot and can affect freshwater and saltwater fishes throughout the world (Stoskopf, 1993). *P. fluorescens* releases extracellular proteases upon invasion of the fish host (Li and Flemming, 1968), and morbidity can be quite high. Outbreaks of bacterial tail rot have been reported in goldfish, silver carp, bighead, tench, grass carp, black carp, golden shiner, rainbow trout, European eels, paradise fish, and other labyrinth fishes. Egusa (1992) reviewed *Pseudomonas* diseases in fish and indicated that, in the United States, the disease termed *Pseudomonas* septicemia, due to infection by bacteria related to *P. fluorescens* (AFS-FHS, 1975), is seen in comparatively large numbers in warm-water fish.

Adverse effects associated with *P. fluorescens* in fish species often appear to be linked to stress from transportation or cultivation of fish. For example, *P. fluorescens* has been associated with disease in the cultivation of rainbow trout, *Oncorhynchus mykiss* (Barros et al., 1986), Atlantic salmon, *Salmo salar* (Carson and Schmidtke, 1993), chinook salmon, *Oncorhynchus tshawytscha* (Newbound et al., 1993), sea bream, *Evynnis japonica* (Kusuda et al., 1974), bighead carp, *Aristichthys nobilis*, and silver carp, *Hypophthalmichthys molitrix* (Petrinec et al., 1985), catfish and carp (Gatti and Nigelli, 1984), tench (Ahne et al., 1982), and tilapia species (Okaeme, 1989; Miyashita, 1984; Miyazaki et al., 1984).

Barker et al. (1991) found that exposure of high numbers of *P. fluorescens* to egg surfaces of rainbow trout (*Oncorhynchus mykiss*) during the initial stages of incubation poses a threat to egg survival. Conversely, *P. fluorescens* was not pathogenic when injected into brown trout (Smith and Davey, 1993) or silver mullet fish (*Mugil curema*) (Alvarez and Conroy, 1987).

P. fluorescens has also been implicated in pathogenicity to some invertebrates. James and Lighthart (1992) determined an LC_{50} for the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar larvae of a coleopteran insect (*Hippodamia convergens*) (4.8×10^{9}, 2.8×10^{10}, 3.9×10^{9}, and 3.2×10^{11} CFU/ml, respectively) and concluded that *P. fluorescens* is a weak bacterial pathogen. *P. fluorescens* has also been reported to be associated with pathogenicity in the mosquitoes *Culex quinquefasciatus*, *Anopheles stephensi*, and *Aedes aegypti* (Murty et al., 1994) and in the field slug *Deroeras reticulatum* (Wilson et al., 1994). However, Genthner et al. (1993) studied the effects of *P. fluorescens* on eastern oysters (*Crassostrea virginica*) and found no signs of infectivity or pathogenicity.

Pathogenicity to plants

P. fluorescens is generally considered to be a saprophyte rather than a plant pathogen (Bradbury, 1986), although Ormrod and Jarvis (1994) considered it to be an opportunistic pathogen causing soft rot in plants. *P. fluorescens* biovar 2 (*P. marginalis*), however, is actively pectinolytic, causing soft rot of various plants, and is considered a plant pathogen (Tsuchiya et al., 1980; Hildebrand, 1989; Membre and Burlot, 1994; Brock et al., 1994). Bradbury (1986) recognised three pathovars in *P. marginalis* which cause soft rot in a wide range of vegetables and other plants.

A number of studies have reported adverse effects associated with *P. fluorescens* and plants (Gaudet et al., 1980; Anson, 1982; Hwang et al., 1989; Richardson, 1993; Ozaktan and Bora, 1994). Tranel et al. (1993) found that *P. fluorescens* strain D7 inhibited root growth of downy brome (*Bromus tectorum*) by production of a phytotoxin. Sellwood et al. (1981) confirmed pathogenicity experimentally for an atypical *P. fluorescens* biotype I on chicory plants and suggested that the group *P. fluorescens* does not solely comprise saprophytes. However, other studies have found no adverse effects on plants from inoculations with *P. fluorescens* (Arsenijevic, 1986; Arsenijevic and Balaz, 1986; Surico and Scala, 1992). At present, the epidemiology of pathogenic strains of *P. fluorescens* is not well understood (Hildebrand, 1994).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. fluorescens* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated that the *pel* gene encoding production of pectate lysase (an enzyme which contributes ability to cause soft rot in plants) is well conserved in fluorescent pseudomonads, and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Liao further indicated that saprophytic strains of *P. fluorescens* can be induced to become pathogenic and raised a concern about the safety of using the species as a biocontrol agent.

23.4 *P. fragi*

Pathogenicity to animals

No reports were found of *P. fragi* as an animal pathogen.

Pathogenicity to plants

No reports were found of *P. fragi* as an plant pathogen.

23.5 *P. putida*

Pathogenicity to animals

P. putida can infect a variety of animals including goats (Hungerford, 1990), koala (Ladds et al., 1990), turkey (Ononiwu, 1980) and fish (Kusuda and Toyoshima, 1976). However, because it is unable to grow at elevated temperatures like that of the human body (Palleroni, 1992a), it is unlikely to be more than a rare opportunistic pathogen for warm-blooded animals.

Kusuda and Toyoshima (1976) reported *P. putida* to be a pathogen to cultivated yellowtail fish. However, there have been no reports on the epizootiology, symptoms, or histological or pathological findings, and the disease has not been well-defined (Egusa, 1992). Austin and McIntosh (1991) considered *P. putida* to be one of a variety of gram-negative bacteria pathogens of potential concern to farmed and wild fish. *P. putida* has also been associated with pathogenicity in the snail, *Biomphalaria glabrata* (Cheng, 1986), the crayfish (Boemare and Vey, 1977), and the olive fly (Haniotakis and Avtizis, 1977).

Pathogenicity to plants

. *P. putida* was included in the *Guide to Plant Pathogenic Bacteria* solely because its multiplication in the rhizosphere of paddy rice plants has been implicated in “suffocation disease”, which arises under conditions of poor drainage (Bradbury, 1986). Studies have reported that *P. putida* is not pathogenic to mushrooms (Ozakatan and Bora, 1994) or crucifer plants (Shaw and Kado, 1988).

Liao (1991) has cautioned that, at present, it is not known for sure that *P. putida* strains are non-pathogens and do not cause deleterious effects on plants. Liao indicated the *pel* gene encoding production of pectate lysase (an enzyme which contributes ability to cause soft rots in plants) is well conserved in fluorescent pseudomonads and may exist and remain repressed in certain strains or species which exhibit non-pectolytic phenotypes under laboratory conditions. Homologous sequences were found in strains of *P. putida,* and Liao raised a concern about the safety of using *P. putida* as a biocontrol agent.

23.6 *P. syringae*

Pathogenicity to animals

No reports were found of *P. syringae* as an animal pathogen.

Pathogenicity to plants

P. syringae is principally an assemblage of foliar pathogens, although it occurs as both pathogenic and epiphytic (non-pathogenic) strains. The species has a broad range of potential plant hosts (Table 5). Pathogenic strains can exhibit both pathogenic (i.e. disease-causing) and epiphytic behaviours on susceptible hosts (Crosse, 1959). The initiation of infection results when a threshold level of bacteria is

reached on the leaf surface; in the case of *P. syringae* pv. *syringae* this is reported to be 10^4 cfu gm^{-1} tissue (Hirano and Upper, 1983).

The association between rain and the onset of foliar blights caused by *P. syringae* is well recognised. Rain appears to stimulate the differential growth of pathogenic *P. syringae* isolates from the heterogeneous populations (pathogenic and non-pathogenic strains). Rain-triggered growth of *P. syringae* results in the establishment of large pathogenic populations required for disease development (Hirano and Upper, 1992).

There appears to be a distinctive set of symptoms associated with each causal agent. *Pseudomonas syringae* pv. *savasatoni* incites tumourous outgrowths on stems and leaves of oleander and olive under natural conditions. These symptoms have been found to be associated with the production of the auxin, indole acetic acid (IAA), in tissues infected with the bacterium. Furthermore, chlorosis, a common symptom when plants are infected by a number of pathogens belonging to the *P. syringae* group, is indicative of production of a toxin. For example, halo blight of beans caused by *P. syringae* pv. *phaselicola* is mediated by the toxin, phaseolotoxin. Other phytopathogenic pseudomonads producing toxins are illustrated in Table 6.

The *Dictionary of Natural Products* (Chapman and Hall, 1995) lists the following toxins produced by various strains of *P. syringae*: 1H-Indole-3-carboxaldehyde, octicidin (phytotoxin), phaseolotoxin (phytotoxin), N-Phosphosulfamylornithine (phytotoxin), syringomycin (phytotoxin), syringostatin A (phytotoxin), syringostatin B (phytotoxin), syringotoxin B (phytotoxin), tagetitoxin (phytotoxin), coronafacic acid (induces chlorosis in plants), halotoxin (phytotoxin), tabtoxin (phytotoxin). Coronatine (phytotoxin) is also produced by certain strains of *P. syringae* (Cuppels and Ainsworth, 1995). Gross (1985) determined that syringomycin production was stimulated by iron and suppressed by inorganic phosphate, that production occurred between 15 and 27°C, and that a slow growth rate of *P. syringae* favours toxin production.

Table 5

Range of plant species susceptible to infection with *P. syringae*

Plant (genus, species)	***P. syringae* pathovar**
Acer spp.	aceris
Aesculus indica	aesculi
Antirrhinum majus	antirrhini
Apium graveolens	apii
Beta spp., *Heleanthus annuus, Tropaeolum majus*	aptata
Avena sativa, Hordeum vulgare, Triticum aestivum	atrofaciens
Agropyron spp., *Agrostis* spp., *Bromus* spp., *Dlymus* spp., *Festuca* spp., *Lolium* spp., *Phleum pratense*, *Stipa* spp.	atropurpurea
Corylus avellana	avellanae
Berberis spp.	bereridis
Cannabis sativa	cannabina
Ceratonia siliqua	ciccaronei
Avena spp., *Arrhenatherum elatius, Calamogrostis montanensis, Deschampsia caespitosa, Koeleria cristata, Phelum partense, Triticum* X *Secale, Trisetum spicatum, Zea mays*	coronafaciens
Delphinium spp.	delphinii
Dysoxylum spectabile	dysoxyli
Eriobotrya japonica	eriobotryae
Ficus palmata	fici
Coffea arabica	garcae
Glycine max	glycinea
Helianthus spp.	helianthi
Hordeum vulgare, Triticum aestivum	japonica
Citrullum lanatus, Cucumis spp., *Cucurbita* spp.	lachrymans
Zea mays, Sorghum bicolor	lapsa
Brassica spp., *Raphus sativus*	maculicola
Nicotiana tabacum	mellea
Morus spp.	mori
Prunus spp.	morsprunorum
Myrica rubra	myricae
Oryza sativa	oryzae

Table 5

Range of plant species susceptible to infection with *P. syringae* (cont.)

Plant (genus, species)	***P. syringae* pathovar**
Panicum miliaceum	panici
Malus pumila, Pyrus communis	papulans
Passiflora edulis	passiflorae
Prunus persica	persicae
Phaseolus spp., *Pisum sativum, Pueraria lobata*	phaseolicola
Philadelphus coronarium	philadelphi
Photinia glabra	photiniae
Lathrus spp., *Pisum* spp., *Vicia* spp.	pisi
Allium porrum	porri
Primula spp.	primulae
Protea cynaroides	proteae
Ribes aureum	ribicola
Forsythia intermedia, Fraxinus spp., Ligustrum spp.,	
Nerium oleander, Olea spp., *Nicotiana tabacum*	savastanoi
Sesamum indicum	sesami
Avena sativa, Triticum X *Secale*	striafaciens
many hosts	syringae
Glycine max, Nicotiana tabacum	tabaci
Ambrosia artemisiifolia, Helianthus spp., *Tagetes* spp.	tagetis
Camellia sinensis	theae
Capsicum anum, Lycopersicon esculentum	tomato
Ulmus spp.	ulmi
Viburnum spp.	viburni
Pseudostuga menziesii	

adapted from Bradbury (1986) and Chanway and Holl (1992)

Table 6

Some toxins produced by phytopathogenic *Pseudomonas* sp.

Pseudomonad	Toxin(s)	Mechanism or site of action	Host plant(s)
P. syringae			
pv. *atropurpurea*	coronatine		Italian rye grass
pv. *coronafaciens*	tabtoxin-β-lactam	glutamine synthetase	oat
pv. *garcae*	tabtoxin-β-lactam	glutamine synthetase	coffee
pv. *glycinea*	coronatine/ polysaccharide		soybean
pv. *lachrymans*	extracellular polysaccharides		cucumber
pv. *maculicola*	coronatine		crucifers
pv. *morsprunorum*	coronatine		sour cherry
pv. *phaseolicola*	phaseolotoxin	ornithine transcarbamoylase	bean, kudzu
pv. *savasatoni*	IAA & cytokinins	plant growth regulators	olive, oleander
pv. *syringae*	syringomycins syringopeptins syringotoxins	plasma membrane	peach, maize
pv. *tabaci*	tabtoxin-β-lactam	glutamine synthase	tobacco
pv. *tagetis*	tagetitoxin	chloroplastic RNA polymerase	marigold
pv. *tomato*	coronatine		tomato
P. tolaasii	tolaasin	plasma membrane	mushroom

taken from Durbin (1996)

23.7 *P. tolaasii*

Pathogenicity to animals

No reports were found of *P. tolaasii* as an animal pathogen.

Pathogenicity to plants

P. tolaasii causes Brown blotch (bacterial blotch), the most common bacterial disease of the commercial button mushroom, *Agaricus bisporus* (Howard et al., 1994). This disease can result in serious economic losses. *P. tolaasii* has also been found to cause disease in the oyster mushroom, *Pleurotus ostreatus,* and the shiitake mushroom, *Lentinus edodes* (Suyama and Fujii, 1993).

P. tolaasii produces a haemolytic lipodepsipeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agariculs bisporus* (Rainey et al., 1993). Tolaasin is phytotoxic when infiltrated into tobacco leaves (Rainey et al., 1991). Synthesis of tolaasin is chromosomally determined, and it is known that at least five genetic loci are required for tolaasin synthesis (Rainey et al., 1993).

24. Interactions with and effects on other organisms in the environment

24.1 *P. aeruginosa*

Certain *P. aeruginosa* strains are antagonistic to plant pathogens such as damping-off fungi (Bradbury, 1986; Buysens et al., 1994). Duffy and Defago (1995) found that clinical and plant isolates of *P. aeruginosa* suppressed root diseases of cucumber, maize and wheat caused by soilborne fungi *Gaeumannomyces graminis* var. *tritici*, *Phomopsis sclerotiodes*, *Pythium ultimum* and *Rhizoctonia solani*. A soil isolate of *P. aeruginosa* suppressed foliar disease on wheat caused by *Septoria tritici* (Flaishman et al., 1990).

P. aeruginosa can have a synergistic effect on the survival of salmonellae, enabling them to survive more than 140 days in double-distilled water (Warburton et al., 1994). It has also been suggested that *P. aeruginosa* may act synergistically with pectolytic bacteria that colonise vegetables, such as *P. marginalis* (*P. fluorescens*) and *Erwinia cartovora* (Bradbury, 1986). A protective immunity against *P. aeruginosa* infection has been reported in mice vaccinated with heat-killed *Lactobacillus casei* (Miake et al., 1985).

P. aeruginosa is known to produce 1-phenazinecarboxamide (the amide of 1-phenazinecarboxylic acid), which is active against some phytopathogenic fungi and *Candida albicans.* A related compound, 1-phenazinol, which is active against gram-positive bacteria and fungi, and which shows some viral activity, is also produced by *P. aeruginosa*. 1-phenazinol has an LD_{50} of 500 mg/kg in mice dosed intraperitoneally. Pyoluteorin and its 3'-nitro derivative are produced by *P. aeruginosa*. Both compounds have antibacterial, antifungal and herbicidal properties. The LD_{50} of the pyoluteorin to mice is 125 mg/kg (Chapman and Hall, 1995). The antibiotic, 2-heptyl-4-hydroxyquinoline N-oxide, is a metabolite of *P. aeruginosa* and is a potent 5'- lipoxygenase inhibitor, with an LD_{50} of 40 mg/kg in mice dosed intraperitoneally (Chapman and Hall, 1995).

24.2 *P. chlororaphis*

P. chlororaphis has been widely investigated for its ability to enhance plant growth through suppression of deleterious root-colonising bacteria. Compounds known as siderophores are produced by *P. chlororaphis*. These compounds chelate iron, thereby depriving certain root-colonising plant pathogens of iron necessary for their growth (Smirnov et al., 1991).

Many studies have indicated that *P. chlororaphis* has the ability to suppress plant disease. For example, *P. aureofaciens* has been investigated as a biocontrol agent to suppress take-all, the wheat root fungal disease. The ability of *P. aureofaciens* to inhibit *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all, was demonstrated *in vitro* and *in vivo* (Harrison et al., 1993). It is believed that disease suppression is largely due to the production of phenazine antibiotics (Thomashow and Pierson, 1991). Carruthers et al. (1995) tested the ability of *P. aureofaciens* to suppress root rot of *Asparagus officinalis* caused by *Phytophthora megasperma* var. *sojae*. *P. aureofaciens* significantly reduced the level of infection and disease severity. Other tests suggested that *P. aureofaciens* had a direct growth stimulatory effect on asparagus, independent of antibiotic production (Carruthers et al., 1995). Berg and Ballin (1994) found *P. chlororaphis* inhibited the growth of the phytopathogenic fungus *Verticillium dahliae.*

When Douglas fir seed was inoculated with *P. aureofaciens,* and grown in pasteurised soil, shoot biomass increased significantly when compared with non-inoculated controls (Chanway and Holl, 1992). *P. aureofaciens* has been found to inhibit mycelial growth of *Rhizoctonia solani* in dual culture between 15 and 30°C (Lee et al., 1990). Inoculation of rice seeds was found to control rice sheath blight in the early growth stages, and seedling blight caused by *R. solani*, *Fusarium moniliforme* and *Pythium ultimum* was suppressed by seed treatment and soil incorporation of *P. aureofaciens* (Lee et al., 1990). In another experiment, the emergence of sweet corn seedlings from soil infested with *Pythium ultimum* was greatly enhanced by coating the seed with *P. aureofaciens* (Mathre et al., 1994). *P. aureofaciens* has also been evaluated for its ability to suppress *Pythium ultimum* damping off of cucumber seedlings (Sugimoto et al., 1990).

P. aureofaciens was antagonistic to *Clavibacter michiganensis* subsp. *sepedonicus,* the bacteria implicated in potato ring rot in greenhouse trials with potato seedlings (de la Cruz et al., 1992). *P. aureofaciens* significantly reduced populations of, and infection by, the ring rot bacteria (de la Cruz et al., 1992). Fukui et al. (1994) investigated the relationship between pericarp colonisation by *Pythium ultimum* in sugar beets and the growth of pseudomonads in the spermosphere. They found a positive correlation between the incidence of pericarp colonisation by *Pythium ultimum* and the length of the lag phase of the strain used to inoculate the seeds. England et al. (1993) investigated the nodulation of whitebean (*Phaseolus vulgaris* L.) by *Rhizobium phaseoli* in the presence of *P. aureofaciens*. No significant difference was found in the numbers of nodules produced in the presence of *P. aureofaciens* as a result of the symbiotic relationship between *Rhizobium phaseoli* and whitebean roots in vermiculite.

P. chlororaphis was observed to interfere with the growth of shiitake mushrooms in field experiments with shiitake cultivated logs (Raaska and Mattila-Sandholm, 1991). Siderophores were produced, however the addition of iron to *in vitro* cultures did not entirely neutralize the growth inhibition of mycelia by *P. chlororaphis.* It was concluded that although iron-binding plays an important role, it is not the only factor involved in the inhibition of shiitake by *P. chlororaphis* (Raaska and Mattila-Sandholm, 1991). A siderophore extracted from *P. aureofaciens* was found to inhibit uptake of ferric iron by maize and pea, and the synthesis of chlorophyll in these plants was reduced (Becker et al., 1985).

P. aureofaciens is reported to produce an antibiotic-like compound in iron-rich conditions that inhibits the growth of the plant fungal pathogen *Aphanomyces euteiches* (Carruthers et al., 1994). Mazzola

et al. (1992) suggested that the production of phenazine antibiotics contributes to the ecological competence of *P. aureofaciens*, and that reduced survival of strains unable to produce the antibiotics is due to diminished ability to compete with the resident microflora. Thomashow et al. (1990) found that suppression of take-all is related directly to the presence of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of wheat. In another experiment, 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine were also found to be responsible for take-all suppression in wheat (Pierson and Thomashow, 1992). Pyrrolnitrin [3-chloro-4-(3-chloro-2-nitrophenyl)-1H-pyrrole] is an antifungal compound produced by *P. chlororaphis*; its LD50 in mice dosed orally is 1 g/kg (Chapman and Hall, 1995). The antifungal compound, 1,3,6-trihydroxy-2,4-diacetophenone, has also been isolated from culture media (Harrison et al., 1993).

24.3 *P. fluorescens*

P. fluorescens has been recognised as beneficial to plant growth (Weller and Cook, 1986; Kloepper et al., 1988). It can enhance plant growth through production of siderophores, which efficiently complex environmental iron, making it unavailable to other components of the soil microflora. Increased plant yields achieved through the inoculation of plant roots have been mimicked by the application of the siderophore, pseudobactin, isolated from *P. fluorescens*. Antibiotic production by *P. fluorescens* has been recognised as an important factor in its ability to suppress phytopathogens. *P. fluorescens* has also been found to significantly promote nodulation, growth and nitrogen accumulation in faba beans (*Vicia faba*) (Omar and Abd-Alla, 1994). Heat-killed cells had no effect.

Certain strains of *P. fluorescens* can promote the formation of ice crystals in water at temperatures near 0°C (Lindow and Panopoulos, 1988; Lindow, 1992). Large populations of these ice^+ bacteria on plant surfaces can cause frost injury. Only 0.01 to 40% of the total bacteria on plant surfaces are sufficient to cause frost injury. In the absence of these bacteria, water on plants can cool to -40°C.

Smith and Davey (1993) found that *P. fluorescens* strains were able to inhibit *Aeromonas salmonicida* that was isolated from Atlantic salmon with furunculosis. Pre-smolts asymptomatically infected with *A. salmonicida* and bathed in a solution containing *P. fluorescens* strains were less likely to develop stress-induced furunculosis than non-treated fish. It was concluded that *P. fluorescens* inhibits *A. salmonicida* by competing for free iron, and that it protects against stress-induced furunculosis by inhibiting *A. salmonicida* on external locations. Kimura et al. (1990) found that a strain of *P. fluorescens* biovar I (46NW-04) isolated from the aquatic environment produced an antiviral substance that was effective against fish viruses.

24.4 *P. fragi*

Monitoring of microbial flora succession on minced lamb meat revealed that *P. fragi* was the dominant climax species (Drosinos and Board, 1995). Another study indicated that *P. fragi* dominated the flora on lamb carcasses at both 7 and 30°C (Prieto et al., 1992).

24.5 *P. putida*

P. putida is very common in soils and plant rhizospheres, where it seems to have a stimulating effect on plant growth (Palleroni, 1984). *P. putida* has been shown to suppress a variety of plant pathogens and to reduce the incidence of plant disease (Liao, 1989; Gamliel and Katan, 1993; Duijff et al., 1994; Freitas et al., 1991; Defago and Hass, 1990). This may be due in part to its inhibition of plant pathogenic microorganisms by sequestering iron or producing metabolites with antibiotic properties. Formation of a

siderophore complex by the plant may also be involved (Defago and Hass, 1990). Siderophore-mediated competition for iron was indicated as the mechanism of suppression of Fusarium wilt of carnation by *P. putida* (WCS358r) (Duijff et al., 1994) and suppression of phytopathogens to winter wheat (Freitas et al., 1991).

Al-Achi et al. (1991) found that, when introduced as pairs into irradiated, sterile soils, a *P. fluorescens* strain prevented optimum colonisation by a *P. putida* strain. The addition of *P. putida* to sterile soil already populated by *P. fluorescens* impeded growth of *P. putida* in that soil. However, adding *P. fluorescens* to soil populated by *P. putida* did not prevent growth of *P. fluorescens* and caused a decrease in *P. putida*. These results suggested competition for similar niches in soils, and that *P. fluorescens* was the more competitive species studied.

24.6 *P. syringae*

Some strains of *P. syringae* have the ability to cause ice nuclei to form at temperatures just below 0°C, thus inducing freezing injury to susceptible plants and allowing disease development to occur (Lindow, 1983). Nutritional starvation for nitrogen, phorphorous,sulphur or iron at 32°C, followed by a shift to 14-18°C, led to the rapid induction (from non-detectable to 100% in 2 to 3 h) of type I ice nuclei (Nemecek-Marshall et al., 1993).

Replacement series experiments on bean leaves between *P. syringae* and epiphytic *P. fluorescens*, *Pantoea agglomerans* (*Erwinia herbicola*), *Stenotrophomonas maltophilia* (*Xanthomonas maltophilia*) and *Methylobacterium organophilum* have demonstrated that the epiphytes were all capable of higher levels of coexistence with *P. syringae* than was observed with another *P. syringae* strain. The level of coexistence with the epiphytes was inversely correlated with the ecological similarity of the strains and with a differential preference for amino acids, organic acids and carbohydrates (Wilson and Lindow, 1994).

The invasion and exclusion abilities of 29 strains of *P. syringae* were studied on leaves in 107 pairwise combinations in which each strain was inoculated on day 0, and the second (challenge) was inoculated on the same leaf on day 3 (Kinkel and Lindow, 1993). The presence of an established population often significantly reduced the growth of the second strain when quantified on day 6; successful invaders (challenge) were significantly less likely to exclude challenge populations than were non-successful invaders. Hirano and Upper (1993) determined that an introduced antibiotic-resistant strain of *P. syringae* spread but did not persist when applied to bean plants grown in the field; it was concluded that the introduced strain was less fit than the pool of indigenous species. Competition between indigenous soil bacteria and single cells of *P. syringae* pv. *syringae* engineered with bioluminescence genes from *Vibrio harveyi* can be monitored using charge-coupled enhanced microscopy (Silcock et al., 1992).

Defreitas et al. (1993) determined that *P. syringae* R25 inoculated on field peas (*Pisum sativum*) did not affect plant growth in plastic growth pouches but, in soil, did inhibit nitrogenase activity of nodules formed by indigenous rhizobia; *P. syringae* R25 inhibited the growth of field beans (*Phaseolous vulgaris*) in both plastic growth pouches and in soil. When peas were inoculated with both *P. syringae* R25 and *Rhizobium leguminosarum*, there was an increase in plant biomass in growth pouches but no effect was observed in soil; when beans were inoculated with both *P. syringae* R25 and *Rhizobium phaseoli*, there were severe deleterious effects on seedling emergence, plant biomass and nodulation in both growth pouches and soil.

Table 7

Phytopathogenic strains of *P. syringae* containing plasmids

Pathovar	**Reference**
P. syringae pv. *angulata*	Piwowarski and Shaw, 1982
P. syringae pv. *atrpurpurea*	Sato et al., 1983
P. syringae pv. *coronafaciens*	Piwowarski and Shaw, 1982
P. syringae pv. *glycinea*	Curiale and Mills, 1983
P. syringae pv. *lachrymans*	Coplin, 1989
P. syringae pv. *papulans*	Burr et al., 1988
P. syringae pv. *phaseolicola*	Quant and Mills, 1984
P. syringae pv. *savastanoi*	Comai et al., 1982
P. syringae pv. *striafaciens*	Beck-Von Bodmann and Shaw, 1987
P. syringae pv. *syringae*	Gonzales et al., 1984
P. syringae pv. *tabaci*	Obukowicz and Shaw, 1983; 1985
P. syringae pv. *tomato*	Denny, 1988; Bender and Cooksey, 1986

24.7 *P. tolaasii*

P. tolaasii produces a haemolytic lipodepsipeptide toxin, tolaasin, which is a potent biosurfactant (Hutchison and Johnstone, 1993) and the primary disease determinant of its pathogenicity to the cultivated mushroom, *Agariculs bisporus* (Rainey et al., 1993). Tolaasin is also active against a range of basidiomycetes and gram-positive bacteria (Rainey et al., 1991).

The nematode *Caenorhabditis elegans* is reported to decrease the spread of *P. tolaasii* in mushroom growth chambers (Grewal, 1991). *P. fluorescens* biovar *reactans* was frequently isolated from the gut of *C. elegans* along with mushroom sporophores. All the isolates of *P. fluorescens* biovar *reactans* isolated from nematodes were antagonists to *P. tolaasii*. It was suggested that, as *C. elegans* selects *P. fluorescens* biovar *reactans* rather than *P. tolaasii* as a food, it probably spreads the antagonist in the mushroom crop and may contribute to the control of mushroom blotch (Grewal, 1991). *P. fluorescens* has also been described by other researchers as antagonistic to *P. tolaasii* (Khanna and Olivier, 1989; Munjal et al., 1989; Nair and Fahy, 1972). Nair and Fahy (1972) reported *Enterobacter aerogenes* to be antagonistic to *P. tolaasii*.

Thorn and Tsuneda (1992) report that 23 species of wood-decay basidiomycetes attacked or lysed *P. tolaasii* when tested. Attack took the form of increased hyphal branching within the bacterial colonies, often preceded by directional growth toward them.

25. Ability to form survival structures (e.g. spores, sclerotia)

Pseudomonads are asporogenous, that is, they do not form spores or other survival structures. Pseudomonads are, however, pleomorphic and represent a tremendously diverse group of strains able to tolerate extreme environmental conditions, including the extremes of temperature.

Bacteria that do not form survival structures like spores and cysts are suspected to have other survival strategies. A number of researchers have reported the existence of dwarf or ultramicrobacteria in nutrient-stressed environments (Rosak and Colwell, 1987). These cells have been described from seawater (Amy and Morita, 1983) and soil (Casida, 1977). Cells are able to develop to their full size, once exposed to an abundant supply of nutrients.

26. Routes of dissemination, physical or biological

Physical

Pseudomonads may be disseminated by air or water currents. For example, Trevors et al. (1990) used soil-core microcosms to study the movement of a *P. fluorescens* isolate through soil planted with wheat and unplanted. In the absence of ground water flow, limited movement was detectable along the soil column planted with wheat, while no movement was detected in the unplanted soil. In contrast, movement of the strain through the column was dependent on the flow rate of the water and the number of times the columns were flushed through. Water flow also affected the distribution of the inoculant along the wheat roots. Bacterial cell size has been related to the movement of cells through a soil column, with smaller bacterial cells (< 1.0µm) moving fastest through the column (Gannon et al., 1991).

Rain may also be an important source of inoculum and means of dispersal for pseudomonads. Rain splash has been attributed to move pseudomonads colonising leaf surfaces down the plant canopy and into the soil (Hirano and Upper, 1992; Butterworth and McCartney, 1992; McCartney and Butterworth, 1992). Large drops of artificial rain were more effective in dispersing bacteria than smaller drops (Butterworth and McCartney, 1992). Humidity correlated positively with the consequent survival of pseudomonads dispersed by rain splash (McCartney and Butterworth, 1992). However, dispersal is short range (one or a few metres) (Constantidou et al., 1990). Pseudomonads (including *P. syringae* Ice^+ strains) have been found to leave plant surfaces in an aerosol-stable state and enter the troposphere during dry, warm weather (Lindemann et al., 1982; Lindemann and Upper, 1985). They are then transported and washed downwards during rainfall (Constantidou et al., 1990).

Biological

Pseudomonads are motile bacteria characterised by the presence of at least one flagellum. While there is no convincing evidence that the bacteria are flagellated in soil (Stotzky et al., 1991), flagella appear to confer increased epiphytic fitness on *P. syringae* strains in association with moisture on leaf surfaces (Haefele and Lindow, 1987). The potential for certain fluorescent pseudomonads to colonise plant surfaces has been attributed to the presence of pili (Vesper, 1987; de Groot et al., 1994), surface charge properties (James et al., 1985), the production of agglutinin, a glycoprotein complex, released from root surfaces (Anderson, 1983), and the ability of certain saprophytic pseudomonads to adhere to the agglutinin of specific plant species (Glandorf et al., 1993; 1994).

Earthworms moving through soil have been implicated in the dissemination of bacteria over short distances. As well, Johnson et al. (1993) have demonstrated the ability of honey bees to disseminate a biological control strain of *P. fluorescens* used against the fireblight pathogen, *Erwinia amylovora,* in apple and pear blossoms. Honey bees carrying approximately 10^4 to 10^6 cfu per bee effectively inoculated fruit tree blossoms with bacteria.

27. Containment and decontamination

Containment plans have been proposed for microbial releases, although few of them have been used, and their efficacy is yet to be demonstrated. It is likely to be difficult to eliminate all the bacteria from a site of introduction. Many of the proposed chemical treatments have gross rather than localised effects; hence their application may have considerable impact on the natural flora, fauna and microflora at the site. Pseudomonads will colonise many laboratory and hospital disinfectants, and may exhibit broad spectrum resistance to a number of widely used antibiotics. Disinfectants based on quaternary ammonium compounds and chlorhexidine solutions have been found to be contaminated with pseudomonads. Disinfectant contaminants include *P. aeruginosa, P. fluorescens,* and *P. cepacia* (Bergen, 1981).

P. putida strains that degrade alkylbenzoates have been modified to carry a fusion of the P (lac) promoter to the gef gene, which encoded a killing protein (Molin et al., 1993; Ramos et al., 1994). Expression from P (lac) was controlled through a regulatory cascade, so that P (lac) was switched on or off by the absence or presence of alkylbenzoates respectively. Similar uncontained strains were also constructed and tested as a control. Contained and uncontained strains were genetically stable, and their survival and functionality in soil microcosms were as expected. Both contained and uncontained strains survived well in soils supplemented with alkylaromatics, whereas survival of the contained strain in soil microcosms without methylbenzoates was markedly reduced in contrast to the control strain, which survived in these soils in the absence of alkylbenzoates (Jensen et al., 1993; Ronchel et al., 1995).

28. Description of detection and monitoring techniques, including specificity, sensitivity and reliability

28.1 Techniques employed in the laboratory and/ or environment for detecting the presence of, and for monitoring, numbers of the organism

Information on detection and monitoring techniques is provided in this information element as well as in information element 2 and Table 8. Each of the well-described detection methods has limitations as well as advantages for enumeration and/or detection (Drahos, 1992). For example, under certain conditions an approach which provides reasonable sensitivity by culturing a microorganism (e.g. the viable plate count) may give reliable data for culturable populations. Furthermore, many approaches are complementary; methods utilising nutritional, antibiotic and enzymatic markers rely on the ability of the target organism to express the marker genes during the selection or reculturing process. However, expression of these traits may not always be optimal, for instance under conditions of severe environmental stress. In these situations, a direct method of detection could be used.

Selective plating

Selective plating has been used widely in combination with selectable phenotypes based on antibiotic resistances (often spontaneous mutations) (Compeau et al., 1988; Fredrickson et al., 1989; Thompson et al., 1990) or introduced genes such as *xylE* (Winstanley et al., 1989; Morgan et al., 1989) l*acZY* (Cook et al., 1991; Drahos et al., 1988), *lux* (Shaw and Kado, 1986) and *mer* gene (Iwasaki et al., 1993; 1994). A number of these genes have been used for marking and tracking pseudomonads. It is important to ensure that the marker is not found in the indigenous microflora of the environment to which the microorganism will be introduced.

Pseudomonads appear to be highly culturable on laboratory media and may be isolated from environmental samples using viable plating (Drahos, 1992). Generally, 1 g of environmental sample is homogenised or shaken in 9 ml of an appropriate dilutent such as ¼ strength Ringer's solution or physiological saline. The homogenate is serially diluted 1 in 10, 100 µl aliquots spread onto selective agar, and the plates incubated at 28°C. A number of selective media are available commercially, such as *Pseudomonas* selective agar (Oxoid) and *Pseudomonas* agar F (Difco). Both media have a low iron content, promoting the production of the iron-chelating, fluorescent siderophores. Selective agars are supplemented with antibiotics. A commercially prepared cocktail of cephalorodine, fucidin acid and cetrimide (Oxoid) is available which may be supplemented with ampicillin and the antifungal agent, cyclohexamide. Microorganisms may be detected at or above a detection limit of 10^2 (i.e. one cell may be detected when a minimum of 100 are present per g of sample) (Trevors and van Elsas, 1989). Sensitivity may be increased by plating larger volumes or by using smaller dilutions, i.e. 1 in 2 instead of 1 in 10.

Most probable number

Most probable number (MPN) methods (Alexander, 1982) have been used to attain greater sensitivity. A serial dilution of the sample is made in an appropriate dilutent to an extinction point (Atlas, 1982). Three to ten replicates of each dilution are made and the pattern of positive and negative scores recorded (i.e. growth or no growth). Statistical tables are used to determine the MPN of microorganisms present in the sample. MPNs like the viable plate count require growth and reproduction of the strains, and may be less accurate since an MPN is established with confidence limits (Jain et al., 1988).

Table 8

Examples of identification and detection techniques

Method	**Reference**	**Sensitivity/reliability**
DNA extraction followed by Polymerase Chain Reaction (PCR)	Stefan and Atlas, 1988	100 *P. cepacia* cells $100g^{-1}$ sediment, against a background of 10 non-target organisms
	Pillai et al., 1991	1 to 10 *E.coli* (with *Tn*5 insert) colony forming unit (cfu)g^{-1} soil
	Tsai and Olson, 1992	3 cells *E.coli* g^{-1} soil; primers directed at 16S rRNA
	Tushima et al., 1995	10 cells g^{-1} water
hybridization using radio-labelled probes	Holben et al., 1988; Stefan and Atlas, 1988	10^3 to 10^4 cells g^{-1} soil
	Jain et al., 1988; Blackburn et al., 1987	10^2 cells g^{-1} soil (similar to viable plate count)
direct microscopy using immuno-fluorescence	Schmidt, 1974; Bohool and Schmidt, 1980; Ford and Olson, 1988	10^6 to 10^7 cells g^{-1} soil
enzyme-linked immunosorbent assays (ELISA)	Morgan et al., 1991; Scholter et al., 1992	10^3 cells g^{-1} soil; $10\text{-}10^2$ cells g^{-1} soil
selective viable plating	Trevors and van Elsas, 1989; Iwasaki et al., 1993,1994	10^2 cfu g^{-1} soil; 1 cfu ml^{-1} water; 10 cfu g^{-1} soil
most probable number (MPN) viable counts	Alexander, 1982; De Leij et al., 1993	$< 10^2$ cfu g^{-1} soil; $<10^1$ cfu g^{-1} soil

Simple chemotaxonomical approach

A simple chemotaxonomical approach which avoids isolation and cultivation of microorganisms has been used. For example, quinone profiles (Hiraishi et al., 1991) or polyamine patterns (Auling et al., 1991) have been used as biomarkers for a survey of pseudomonads (and acinetobacters) in activated sludge from sewage treatment facilities.

Immunological methods

SDS-PAGE coupled with immunological probes have been applied to identify fluorescent pseudomonads of environmental origin (Sorenson et al., 1992). Other possibilities for detecting pseudomonads in environmental samples include the application of phylogenetic probes applied in in situ hybridisations (DeLong et al., 1989), or strain or species-specific monoclonal antibodies labelled with fluorescent dyes (Bohlool and Schmidt, 1980; Conway de Macario et al., 1982). Blair and McDowell (1995) describe an ELISA method for detecting extracellular proteinase of P. fragi.

Microscopic examination and direct enumeration of microorganisms *in situ* can also be used, although this type of approach is not sensitive. To detect one bacterium at a magnification of 1000, the cell density must be 10^6 to 10^7 per g soil. The approach does, however, provide information about the spatial distribution of a strain colonising an environmental substrate, and can be used to enumerate non-culturable microorganisms.

Ramos-Gonzalez et al. (1992) produced highly specific monoclonal antibodies against surface lipopolysaccharides (LPS) of *P. putida* 2440 and developed a semi-quantitative dot blot immunoassay for bacteria in liquid media. This allowed the authors to detect, in complex samples, as few as 100 cells per spot by using peroxidase-conjugated antibody against the antibody that recognised *P. putida* 2440. An intrinsic limitation of this technique is the turbidity of the samples, which may limit maximum assay volume. This assay is also of limited use for bacteria introduced into soils or sediments because of intrinsic fluorescent backgrounds. *P. putida* 2440 (pWWO) released in lake mesocosms have been successfully tracked with monoclonal antibodies (Brettar et al., 1994; Ramos-Gonzalez et al., 1992).

Nucleic acid probes and primers

Nucleic acid probes and/or PCR primers may be used for the detection of gene sequences in the environment. A number of sequence hybridization techniques including Southern, slot-blot, dot-blot, and colony hybridization have been used for environmental isolates. These approaches would be particularly applicable to strains with traits that are not widely distributed throughout the environment under study, and against which specific probes and primers may be designed. The sensitivity of the hybridization approach is variable and for the most part strain-specific. Generally, radioactively labelled probes provide for more sensitivity than non-radioactive probes. Sensitivity can be enhanced using PCR. However, the increased efficiency of the amplified signal obtained by the PCR assay is countered by the inefficient extraction of nucleic acids from environmental samples (Bramwell et al., 1994). For example, soils contain positively charged cations which are sandwiched between layers of clay, and which are able to bind negatively charged nucleic acids, making their retrieval difficult. Caution is required in using PCR as a method for the enumeration of bacteria, as the extreme sensitivity of this procedure renders quantification by target dilution difficult (Drahos, 1992). Thiem et al. (1994) and Zhou and Tiedje (1995) point out the complexity of using molecular techniques for monitoring pseudomonads used for subsurface bioremediation.

Denaturing gradient gel electrophoresis (DGGE) of DNA is a suitable method for those species which are difficult to culture on growth media. This method has been used by Muyzer et al. (1993). Whole DNA is isolated. Using two primers, one with a GC-rich end, a fragment of 16s rDNA is amplified by PCR. This results in a mixture of DNA fragments, equal in size but different in sequence, corresponding to the various organisms in the sample. The mixture is fractionated by DGGE, resulting in one band for each organism type. The bands are sequenced, and based on the sequences, the rRNA-group can be determined.

Polymerase chain reaction (PCR) based sequence amplification

A technique that is finding increasing application for specific identification of microorganisms is the technique referred to as REP-PCR (based on PCR amplification between repetitive sequences commonly found in bacteria). This technique relies on development of adequate databases, but is used with increasing frequency (De Bruijn, 1992). Other approaches are to follow the expressed phenotype attributed to the introduction of a marker gene (e.g. bioluminescent genes) (Prosser, 1994), and to use competitive PCR based on introduction of an internal standard during the PCR amplification (Leser, 1995).

Arbitrary PCR primers

Identification can be facilitated based on the analysis of DNA produced from total DNA, using PCR and arbitrary primers (Welsh and McClelland, 1990; Williams et al., 1990).

Specific PCR primers

P. aeruginosa can be identified using PCR amplification of the 16S-23S rDNA internal transcribed spacer region (Tyler et al., 1995).

28.2 Specificity, sensitivity, reliability

The specificity of identification/monitoring methodologies will generally require some sort of experimental study to demonstrate that the method distinguishes the introduced inoculant from indigenous relatives. An approximate estimate of sensitivity for a number of methods is given in Table 8. However, sensitivity of detection is a function of the organism and of the habitat.

A problem with applying any method of detection is its dependence on extraction efficiencies. Problems are exemplified in soil. Traditionally, bacteria have been recovered from soils through the mechanical shaking of the soil in an appropriate dilutent. The ease of extracting cells or nucleic acids varies between soil types, with extraction efficiencies being higher in sand as opposed to clay-based soils. Strong chemical and physical interactions may occur between microorganisms and the particulate matter of soil. These associations may be ionic, since bacteria are negatively charged and clay soil minerals contain positively charged cations. Dispersion of soil aggregates has been considered important, as entrapment of microorganisms in soil aggregates is considered to be one of the most significant means by which microorganisms are retained in soil (Hopkins et al., 1991). Attempts to disrupt these soil-microbe associations to extract bacteria have utilised homogenisation, chemical dispersants, cation exchange resins, and differential centrifugation (Faegri et al., 1977; Bakken, 1985; MacDonald, 1986; Herron and Wellington, 1990; Hopkins et al., 1991).

Soil is a highly heterogeneous substrate with a non-uniform spatial distribution of bacterial colonies (Wellington et al., 1990). Sampling strategies should consider the variability of the soil matrix

under study; errors attributable to the difficulties of sampling heterogeneous substrates may be compensated for by taking composite samples (Atlas and Bartha, 1981).

Microorganism themselves will also affect the efficiency of extraction of biological molecules such as DNA. For example, bacteria, even those quite closely related, vary in the conditions required for lysis. Hence methodologies aiming to extract the total DNA from soil will selectively recover DNA from isolates that lyse easily, making representative sampling of environmental substrates difficult.

Similar selective pressures apply to viable plating methodologies, since these methods favour the growth of bacteria that readily grow on agar plates under laboratory conditions. Furthermore, all media are selective to some extent, so that certain bacterial species will appear in different proportions, if at all, on different bacteriological agars. Sorheim et al. (1989) compared the populations recovered from soil on three different non-selective media. Bacterial populations exhibiting the same level of diversity were isolated on all media. Each of the media appeared to select for a different population of isolates, with 30% of the population appearing common to all three media. 20% of the isolates recovered from two of the media were distinct to that particular media, and 60% of isolates on the third media were unique to it.

The sensitivity of the viable plate count has been estimated to be 10^2 cfu/g soil (Trevors and van Elsas, 1989). However, this may be improved by combining methods to extract and concentrate the biomass from environmental material prior to plating. Detection limits as low as 10 streptomycete spores per 100 g sterile soil have been demonstrated (Herron and Wellington, 1990).

Pseudomonads are highly culturable on rich media. Their importance may therefore have been overestimated as a result of over-representation on isolation plates (Miller et al., 1990b; Sorheim et al., 1989). Nutritionally limiting isolation media and lower incubation temperatures with longer incubations may allow a greater diversity of bacterial isolates to be recovered from environmental substrates (Miller et al., 1990b).

Ottawa' 92: The OECD Workshop on Methods for Monitoring Organisms in the Environment (OECD, 1994a) includes a review of the monitoring of microorganisms (including *P. aureofaciens*) in the phyllosphere (Bailey et al., 1994) and a review of the different methods available. A companion document, *Compendium of Methods for Monitoring Organisms in the Environment* (OECD, 1994b), contains 39 methods for detecting or monitoring microorganisms, including the following species of *Pseudomonas*: *P. aureofaciens, P. fluorescens, P. putida, P. aeruginosa* and *P. syringae.*

SECTION V – REFERENCES

AFS-FHS (American Fisheries Society, Fish health Section). 1975. Suggested procedures for the detection and identification of certain infectious diseases. U.S. Fish and Wildlife Service, Washington, D.C., 99p.

Ahne, W., Popp, W. and Hoffman, R. 1982. *Pseudomonas fluorescens* as a pathogen for tench. Bulletin of the European Association of Fish Pathologists 2: 56-57.

Aksamit, T.R. 1993. *Pseudomonas* pneumonia and bacteremia in the immunocompromised patient. In: Fick, R.B. Jr. (ed.). *Pseudomonas aeruginosa* the opportunist. Pathogenesis and disease (pp. 177-188). Boca Raton: CRC Press.

Al-Achi, B.J., Platsouka, E. and Levy, S.B. 1991. Competitive colonisation between *Pseudomonas* species in sterile soils. Current Microbiology 23: 97-104.

Alaoukaty, A., Appanna, V.D. and Huang, J. 1992. Aluminum, chromium and manganese detoxification mechanisms in *Pseudomonas syringae* - An X-ray fluorescence study. Microbios 70(282): 13-22.

Alexander, M. 1982. Most probable number method for microbial populations. In: A.L. Page, R.H. Miller and Keeney, D.R. (eds). *Methods of Soil Analysis*, Part 2 (pp. 815-820). ASA and SSA, Madison, WI.

Alford, J.A. and Pierce, D.A. 1961. Lipolytic activity of microoganisms at low and intermediate temperatures. III. Activity of microbial lipases at temperatures below 0° C. Journal of Food Science. 26: 518-524.

Alvarez, J.D. and Conroy, D.A. 1987. The experimental induction of bacterial haemorrhagic septicaemia in silver mullet (*Mugil curema* Val., 1836) held in sea water. Bulletin of the European Association of Fish Pathologists 7: 8-10.

Alvarez-Cohen, L. and P.L. McCarty. 1991. Product toxicity and cometabolic competitive inhibition modelling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Applied and Environmental Microbiology 57: 1031-1037.

Amy, P.S. and Morita, R.Y. 1983. Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. Applied and Environmental Microbiology 45: 1109-1115.

Anderson, A.J. 1983. Isolation from root and shoot surfaces of agglutinins that show specificity for saprophytic pseudomonads. Canadian Journal Botany 61: 3438-3443.

Anderson, M. and Davey, R. 1994. Pseudobacteraemia with *Pseudomonas fluorescens*. Medical Journal Aust. 160: 233-234.

Andreoni, V. and Bestetti, G. 1986. Comparative analysis of different *Pseudomonas* strains that degrade cinnamic acid. Applied and Environmental Microbiology 52: 930-934.

Angelini, N.M. and Seigneur, G.N. 1988. Fin rot in *Rhamdia sapo*. Isolation of the aetiological agents, *Aeromonas hydophila* and *Pseudomonas aeruginosa*, and experimental infection. Revista Argentina de Microbiologia 20(1): 37-48.

Angle, J.S., Levin, M.A., Gagliardi, J.V. and McIntosh, M.S. 1995. Validation of microcosms for examining the survival of *Pseudomonas aureofaciens* (*lac* ZY) in soil. Applied and Environmental Microbiology 61: 2835-2839.

Anson, A.E. 1982. A Pseudomonad producing orange soft rot disease in cacti. Phytopathologische Zeitschrift 103: 163-172.

Arsenijevic, M. 1986. Some weeds as test plants for the pathogenicity of plant pathogenic *Pseudomonas* species. II. Reaction of *Datura stramonium* L. and *Chenopodium album* L. Zastita Bilja 37: 225-229.

Arsenijevic, M. and Balaz, J. 1986. Wheat (*Triticum aestivum ssp. vulgare* (Vill.Host) MacKey) as a test plant for the pathogenicity of *Pseudomonas syringae* pv. *syringae* Van Hall. Zastita Bilja 37: 201-212.

Artenstein, A.W. and Cross, A.S. 1993. Local and disseminated diseases caused by *Pseudomonas aeruginosa*. In: Campa, M., et al. (eds). *Pseudomonas aeruginosa* as an opportunistic pathogen (pp. 224-244). Plenum Press, New York/London.

Assinder, S.J. and Williams, P.A. 1990. The TOL plasmids: determinants of the catabolism of toluene and xylenes. Advances in Microbial Physiology 31: 1-69.

Atlas, R.M. 1982. Enumeration and estimation of microbial biomass. In: Burns, R.G. and Slater, J.H. (eds). *Experimental Microbial Ecology* (pp. 84-102). Blackwell Scientific Publications, Oxford, UK.

Atlas, R.M. and Baratha, R. 1981. Determination of microbial numbers, biomass and activities. In: *Microbial Ecology: Fundamentals and Applications* (pp.81-132). London: Addison-Wesley, Publishing Company.

Auling, G. 1992. Polyamines, biomarkers for taxonomy and ecology of phytopathogenic and other bacteria. Belgian Journal of Botany. 125: 203-209.

Auling, G., Pilz, F., Busse, H.-J., Karrasch, S., Streichan, M. and Schon, G. 1991. Analysis of the polyphosphate-accumulating microflora in phosphorous-eliminating, anaerobic-aerobic activated sludge systems by using diaminopropane as a biomarker for rapid estimation of *Acinetobacter* spp. Applied and Environmental Microbiology 57: 3585-3592.

Austin, B., and McIntosh, D. 1991. New bacterial fish pathogens and their implications for fish farming. Reviews in Medical Microbiology 2: 230-236.

Babu, G.R.V., Chetty, C.S., Wolfram, J.H. and Chapatwala, K.D. 1994. Microbial metabolism of nitriles and cyanides. Journal of Environmental Science and Health - Part A - Environmental Science and Engineering 29: 1957-1971.

Baggi, G., Boga, M.M., Catelani, D., Galli, E. and Treccani, V. 1983. Styrene catabolism by a strain of *Pseudomonas fluorescens*. Systematic and Applied Microbiology 4: 141-147.

Bailey, M.J., Thompson, I.P. and Lynch, J.M. 1994. Methods for monitoring microorganisms in the phyllosphere, pp. 44-53. In: Ottawa '92: The OECD Workshop on Methods for Monitoring Organisms in the Environment. OECD Environment Monograph No. 90. OECD, Paris.

Bakken, L.R. 1985. Separation and purification of bacteria from soil. Applied and Environmental Microbiology 49: 1482-1487.

Bale, M.J., Fry, J.C. and Day, M.J. 1988. Transfer and occurrence of large mercury resistance plasmids in river epilithon. Applied and Environmental Microbiology 54: 972-978.

Barker, G.A., Smith, S.N. and Bromage, N.R. 1991. Commensal bacteria and their possible relationship to the mortality of incubating salmonid eggs. Journal of Fish Diseases 14: 199-210.

Barrett, E.L., Solanes, R. E., Tang, J.S. and Palleroni, N.J. 1986. *Pseudomonas fluorescens* biovar V: its resolution into distinct component groups and the relationship of these groups to other *P. fluorescens* biovars, to *P. putida*, and to psychrotrophic pseudomonads associated with food spoilage. Journal of General Microbiology 132: 2709-2721.

Barros, G.C., Vianni, M.C.E. and Nogueira, Y.A. 1986. Outbreak of disease in rainbow trout attributed to *Pseudomonas fluorescens*. Revista da Faculdade de Medicina Veterinaria e Zootecnia da Universidade de Sao Paulo 23: 167-173.

B.C.I. (Biotechnology Citation Index), Institute for Scientific Information, CD-ROM. 1991-1995. Philadelphia, U.S.A.

Beattie, G.A. and Lindow, S.E. 1994. Survival, growth and localization of epiphytic fitness mutants of *Pseudomonas syringae* on leaves. Applied and Environmental Microbiology 60(10): 3790-3798.

Beck-Von Bodman, S.B. and Shaw, P.D. 1987. Conservation of plasmids among plant-pathogenic *Pseudomonas syringae* isolates of diverse origins. Plasmid 17: 240-47.

Becker, J.O., Hedges, R.W. and Messens, E. 1985. Inhibitory effect of pseudobactin on the uptake of iron by higher plants. Applied and Environmental Microbiology 49(5): 1090-1093.

Bender, C. L. and Cooksey, D.A. 1986. Indigenous plasmids in *P. syringae* pv. *tomato*: conjugative transfer and role in copper resistance. Journal of Bacteriology 165: 534-541.

Berg, G. and Ballin, G. 1994. Bacterial antagonists to *Verticillium dahliae* kleb. Journal of Phytopathology 141(1): 99-110.

Bergen, T. 1981. Human- and animal-pathogenic members of the genus *Pseudomonas*. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schegel, H.G. (eds). *The Prokaryotes - A Handbook on Habitats, Isolation and Identification of Bacteria*. Springer-Verlag, Berlin.

Berger, T.G., Kaveh, S., Becker, D. and Hoffman, J. 1995. Cutaneous manifestations of *Pseudomonas* infections in AIDS. Journal of the American Academy of Dermatology 32(1): 279-280.

Bestetti, G., Galli, E., Ruzzi, M. Baldicci, G., Zennato, E. and Frontali, L. 1984. Molecular characterization of a plasmid from *Pseudomonas fluorescens* involved in styrene degradation. Plasmid 12: 181-188.

Binnerup, S.J., Jensen, D.F., Thordal, C.H. and Sorensen, J. 1993. Detection of viable, but non-culturable *Pseudomonas fluorescens* DF 57 in soil using a microcolony epifluorescence technique. FEMS Microbiology Ecology 12: 97-105.

Bisbe, J., Gateil, J.M., Puig, J., Mallolas, J., Martinez, J.A., Jimenez de Anta, M.T. and Soriano, E. 1988. *Pseudomonas aeruginosa* bacteremia: Univariate and multivariate analysis of factors influencing the prognosis in 133 episodes. Review of Infectious Diseases 10: 629-635.

Blackburn, J.W., Jain, R.K. and Sayler, G.S. 1987. Molecular microbial ecology of a naphthalene degrading genotype in activated sludge. Environmental Scientific Technology 21: 884-890.

Blair, I.S. and McDowell, D.A. 1995. Detection of extracellular proteinase of *Pseudomonas fragi* by enzyme-linked immunosorbent-assay. Current Microbiology 31(3):180-185.

Bodey, G.P., Bolivar, R., Fainstein, V. and Jajeda, L. 1983. Infections caused by *Pseudomonas aeruginosa*. Review of Infectious Diseases 5: 279-313.

Boelens, J., Woestyne, M.V. and Verstraete, W. 1994. Ecological importance of motilty for the plant growth-promoting rhizo*Pseudomonas* strain ANP15. Soil Biology and Biochemistry 26: 269-277.

Boemare, N. and Vey, A. 1977. Study on bacterial strains isolated from the crayfish *Atlantoastacus pallipes* affected by septicemia and hepato-intestinal diseases. Ann. Hydrobiol. 8: 153-162.

Bohool, B.B. and Schmidt, E.L. 1980. The immunofluorescence approach in microbial ecology. Advances in Microbial Ecology 4: 203-241.

Bottone, E.J. and Perez, A.A. 1993. *Pseudomonas aeruginosa* folliculitis acquired through use of a contaminated loofah sponge: an unrecognised potential public health problem. Journal of Clinical Microbiology 31: 480-483.

Bottone, E.J. and A.A. Perez. 1994. Loofah sponges as reservoirs and vehicles in the transmission of potentially pathogenic bacterial speciies to human skin. Journal of Clinical Microbiology 32: 469-472.

Bowers, J.H. and Parke, J.L. 1993. Colonisation of pea (*Pisum sativum* L.) taproots by *Pseudomonas fluorescens*: effect of soil temperature and bacterial motility. Soil Biology and Biochemistry 25: 1693-1701.

Boyle, A.W., Silvin, C.J., Hassett, H.P., Nakas, J.P. and Tanenbaum, S.W. 1993. Bacterial PCB degradation. In: Microorganisms to Combat Pollution (pp. 161-174). Kluwer Academic Publishers, Dordrecht, Netherlands.

Bradbury, J.F. 1986. Guide to Plant Pathogenic Bacteria. C.A.B. International Mycological Institute. Kew, Surrey, UK.

Bramwell, P.A., Barallon, R.V., Rogers, H.J. and Bailey, M.J. 1994. Extraction of DNA from the phylloplane. In: Akkermans, A.D.L., van Elsas, J.D. and de Bruijin, F.J. (eds). *Molecular Microbial Ecology Manual.* Kluwer Academic Publishers, in press.

Brettar, I., Ramos-Gonzalez, M.I., Ramos, J.L. and Hofle, M.G. 1994. Fate of *Pseudomonas putida* after release into lake water mesocosms: different survival mechanisms in response to environmental conditions. Microbial Ecology 27: 99-122.

Brittingham, M.C., Temple, S.A. and Duncan, R.M. 1988. A survey of the prevalence of selected bacteria in wild birds. Journal of Wildlife Diseases 24(2): 299-307.

Brock, T.D., Madigan, M.T., Martinko, J.M. and Parker, J. 1994. *Biology of Microorganisms* (7th Edition). Prentice Hall, New Jersey.

Brodkin, M.A., Simon, M.P., DeSantis, A.M. and Boyer, K.J. 1992. Response of *Rana pipiens* to graded doses of the bacterium *Pseudomonas aeruginosa*. Journal of Herpetology 26(4): 490-495.

Bukanov, N., Ravi, V.N., Miller, D., Srivastava, K. and Berg, D.E. 1994. *Pseudomonas aeruginosa* corneal ulcer isolates distinguished using the arbitrarily primed PCR DNA fingerprinting method. Current Eye Research 13: 783-790.

Burr, T.J., Norelli, J.L, Katz, B., Wilcox, W.F. and Hoying, S.A. 1988. Streptomycin resistance of *Pseudomonas syringae* pv. *papulans* in apple orchards and its association with a conjugative plasmid. Phytopathology 78: 410-13.

Busse, J. and Auling, G. 1988. Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. Systematic and Applied Microbiology 11: 1-8.

Busse, J., El-Banna, T. and Auling, G. 1989. Evaluation of different approaches for identification of xenobiotic degrading pseudomonads. Applied and Environmental Microbiology 55: 1578-1583.

Butterworth, J. and McCartney, H.A. 1991. The dispersal of bacteria from leaf surfaces by water splash. Journal of Applied Bacteriology 71(6): 484-496.

Butterworth, J. and McCartney, H.A. 1992. Effect of drop size on the removal of *Bacillus subtilis* from foliar surfaces by water splash. Microbial Releases 1: 177-185.

Buysens, S., Poppe, J. and Hofte, M. 1994. Role of siderophores in plant growth stimulation and antagonisms by *Pseudomonas aeruginosa* 7NSK2. In: Ryder, M.H., Stephens, P.M., and Bowen, G.D. (eds). *Improving Plant Productivity with Rhizosphere Bacteria*, pp. 139-141, CSIRO Division of Soils, Glen Osmond, S. Australia.

C.A.B. Abstracts CD-ROM, C.A.B. International. 1972-1995. Wallingford, Oxon, UK.

Cabral, J.P.S. 1995. Viability and respiration activity of *Pseudomonas syringae* cells starved in buffer. Canadian Journal of Microbiology 41(4-5): 372-377.

Carlson, C., Pierson L., Rosen J. and Ingraham J. 1983. *Pseudomonal stutzeri* and related species undergo natural transformation. Journal of Bacteriology 153: 93-99.

Carruthers, F.L., Conner, A.J. and Mahanty, H.K. 1994. Identification of a genetic locus in *Pseudomonas aureofaciens* involved in fungal inhibition. Applied and Environmental Microbiology 60(1): 71-77.

Carruthers, F.L., Shumthomas, T., Conner, A.J. and Mahanty, H.K. 1995. The significance of antibiotic production by *Pseudomonas aureofaciens* Pa147-2 for biological control of *Phytophthora megasperma* root rot of asparagus. Plant and Soil 170(2): 339-344.

Carson, J. and Schmidtke, L.M. 1993. Opportunistic infection by psychrotrophic bacteria of cold-comprised Atlantic salmon. Bulletin of the European Association of Fish Pathologists 13: 49-52.

Casida, L.E. 1977. Small cells in pure cultures of *Agromyces ramosus* and in natural soil. Canadian Journal of Microbiology 23: 214-216.

Castro, C.E. and Belser, N.O. 1990. Biodehalogenation: oxidative and reductive metabolism of 1,1,2-trichloroethane by *Pseudomonas putida* - biogeneration of vinyl chloride. Environmental Toxicology and Chemistry 9: 707-714.

Chakrabarty, A. 1995. Microbial degradation of hazardous chemicals: evolutionary considerations and bioremediative developments. In: *Bioremediation: the Tokyo '94 Workshop* (pp. 71-80). OECD, Paris.

Chamberland, S., L'Ecuyer, J., Lessard, C., Bernier, M., Provencher, P., Bergeron M.G. and the Canadian Study Group. 1992. Antibiotic susceptibility profiles of 941 gram-negative bacteria isolated from septicemic patients throughout Canada. Clinical Infectious Diseases 15: 615-628.

Chanway, C.P. and Holl, F.B. 1992. Influence of soil biota on Douglas-fir (*Pseudotsuga menziesii*) seedling growth: the role of rhizosphere bacteria. Canadian Journal of Botany 70: 1025-1031.

Chanway, C.P. and Holl, F.B. 1993. Ecotypic specificity of spruce emergence-stimulating *Pseudomonas putida*. Forest Science 39: 520-527.

Chapman and Hall, 1995. Dictionary of Natural Products on CD-ROM, Release 4:1. London, UK.

Chatterjee, D.K. and Chakrabarty, A.M. 1983. Genetic homology between independently isolated chlorobenzoate-degradative plasmids. Journal of Bacteriology 153: 532-534.

Chatterjee, A., Kwartz, J., Ridgway, A.E. and Storey, J.K. 1995. Disposable soft contact lens ulcers: a study of 43 cases seen at Manchester Royal Eye Hospital. Cornea 14(2): 138-141.

Cheng, T. 1986. Biological control studies of bacteria associated with moribund *Biomphalaria glabrata* mollusca in the laboratory. Journal of Invertebrate Pathology 47: 219-224.

Cho, J.J., Schroth, M.N., Kominos, S.D. and Green, S.K. 1975. Ornamental plants as carriers of *Pseudomonas aeruginosa.* Phytopathology 65: 425-431.

Christensen, H., Boye, M., Poulsen, L.K. and Rasmussen, O.F. 1994. Analysis of fluorescent pseudomonads based on 23S ribosomal DNA sequences. Applied and Environmental Microbiology 60: 2196-2199.

Comai, L., Surico, G. and Kosuge, T. 1982. Relation of plasmid DNA to indolacetic acid production in different strains of *Pseudomonas syringae* pv. *savasatoni.* Journal of General Microbiology 128: 2157-63.

Compeau, G., Al-Achi, B.J., Plastouka, E. and Levy, S.B. 1988. The survival of rifampicin-resistant mutants of *Pseudomonas putida* and *Pseudomonas fluorescens* in soil systems. Applied and Environmental Microbiology 54: 2432-2438.

Constantinidou, H.A., Hirano, S.S., Baker, L.S. and Upper, C.D. 1990. Atmospheric dispersal of ice-nucleation bacteria: the role of rain. Phytopathology 80: 934-937.

Conway de Macario, E.A., Macario, J.L. and Kandler, O. 1982. Monoclonal antibodies for immunological analysis of methanogenic bacteria. Journal of Immunology 129: 1670-1674.

Cook, R.J., Weller, D.M., Drahos, D.J., Kovacevich, P.A., Hemming, B.C., Barnes, G. and Peirson, E.I. 1991. Establishment, monitoring and termination of field tests with genetically altered bacteria applied to wheat for biological control of take-all. In: Mackenzie, D.R. (ed.), *Biotechnology Field Results*. Academic Press.

Cooksey, D.A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. Annual Review of Phytopathology 28: 201-219.

Coplin, D.L. 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Annual Review of Phytopathology 27: 187-212.

Crosse, J.E. 1959. Bacterial canker of stone-fruits. IV. Investigation of a method for measuring the inoculum potential of cherry trees. Annals of Applied Biology 47: 306-317.

Cuppels, D. and Ainsworth, T. 1995. Molecular and physiological characterization of *Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *maculicola* strains that produce the phytotoxin coronatine. Applied and Environmental Microbiology 61(10): 3530-3536.

Cuppels, D.A. and Kelman, A. 1980. Isolation of pectolytic fluorescent pseudomonads from soil and potatoes. Phytopathology 70: 1110-1115.

Curiale, M.S. and Mills, D. 1983. Molecular relatedness among cryptic plasmids in *Pseudomonas syringae* pv. *glycinea*. Phytopathology 73: 1440-43.

Dandurand, L.M., Morra, M.J., Chaverra, M.H. and Orser, C.S. 1994. Survival of *Pseudomonas* spp. in air-dried mineral powders. Soil Biology and Biochemistry 26: 1423-1430.

De Bruijn, F.J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Applied and Environmental Microbiology 58: 2180-2187.

De Groot, A., Heijnen, I., de Cock, H., Filloux, A. and Tommassen, J. 1994. Characterization of type IV pilus genes in plant growth-promoting *Pseudomonas putida* WCS358. Journal of Bacteriology 176: 642-650.

De la Cruz, A.R., Poplawsky, A.R. and Wiese, M.V. 1992. Biological suppression of potato ring rot by fluorescent pseudomonads. Applied and Environmental Microbiology 58(6): 1986-1991.

De Leij, F.A.A.M., Bailey, M.J., Lynch, J.M. and Whipps, J.M. 1993. A simple most probable number technique for the sensitive recovery of a genetically engineered *Pseudomonas aureofaciens* from soil. Letters in Applied Microbiology 16: 307-310.

De Leij, F.A.A.M., Sutton, E.J., Whipps, J.M. and Lynch, J.M. 1994. Effect of genetically modified *Pseudomonas aureofaciens* on indigenous microbial populations of wheat. FEMS Microbiology Ecology 13: 249-258.

De Leij, F.A.A.M., Sutton, E.J., Whipps, J.M., Fenelon, J.S. and Lynch, J.M. 1995. Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Applied and Environmental Microbiology 61: 3443-3453.

Defago, G. and Hass, D. 1990. Pseudomonads as antagonists of soilborne plant pathogens: modes of action and genetic analysis. In: Bollag, J.M. and Stotsky, G. (eds). *Soil Biochemistry* (pp. 249-291), Vol. 6. Marcel Dekker Inc, New York, N.Y.

Defreitas, J.R., Gupta, V.V.S.R. and Germida, J.J. 1993. Influence of *Pseudomonas syringae* R25 and *P. putida* R105 on the growth the N_2 fixation (acetylene-reduction activity) of pea (*Pisum sativum* L) and field bean (*Phaseolus vulgaris* L). Biology and Fertility of Soils 16(3): 215-220.

DeLong, E.F., Wickham, G.S. and Pace, N.R. 1989. Phylogenetic stains: ribosomal RNA-based probes for the detection of single cells. Science 243: 1360-1363.

Denny, T.P. 1988. Phenotypic diversity in *Pseudomonas syringae* pv. *tomato*. Journal of General Microbiology 134: 1939-48.

Diamond, S.S. and Cadwell, G.A. 1979. Fatal bronchopneumonia and dermatitis caused by *Pseudomonas aeruginosa* in Atlantic Bottle-Nosed Dolphin. Journal of the American Veterinary Medical Association 175: 984-987.

Dick, J.D., Shull, V.B., Karp J.E. and Valentine, J. 1988. Bacterial and host factors affecting *Pseudomonas aeruginosa* colonization versus bacteremia in granulocytopenic patients. EJCCO 24: 547.

Dominik, R.H., Segebade, I.E. and Taenzer, V. 1995. Risk of microbial contamination of iodinated contrast media on multiple use of large-volume bottles. European Journal of Radiology 19(3): 198-205.

Dorn, A. 1976. *Pseudomonas aeruginosa* causes epidemic disease in the milkweed bug, *Oncopeltus fasciatus* Dalla (Insecta, Heteroptera). Experientia 32(5): 599-601.

Doyle, J.D., Short, K.A., Stotsky, G., King, R.J., Seidler, R.J. and Olsen, R.H. 1991. Ecologically significant effects of *Pseudomonas putida* PPO301 (pRO103), genetically engineered to degrade 2,4-dichlorophenoxyacetate, on microbial populations and processes in soil. Canadian Journal of Microbiology 37: 682-691.

Drahos, D.J. 1992. Methods for the detection, identification and enumeration of microbes. In: Andrews, J.H. and Hirano, S.S. (eds). *Microbial Ecology of Leaves.* Springer-Verlag, New York.

Drahos, D.J., Barry, G.F., Hemming, B.C., Brandt, E.J., Skipper, H.D., Kline, E.L., Klupfel, D.A., Hughes, T.A. and Gooden, D.T. 1988. Pre-release testing procedures: U.S. field test of a *lac*ZY-engineered soil bacterium, pp. 181-191. In: Sussman, M., Collins, C.H., Skinner, F.A. and Stewart-Tull, D.E. (eds). *The Release of Genetically-Engineered Microorganisms*. Academic Press, London.

Draper, C.S., Walker, R.D. and Lawler, H.E. 1981. Patterns of oral bacterial infection in captive snakes. Journal of the American Veterinary Medical Association 179: 1223-1226.

Dropulic, L.K., Leslie, J.M., Eldred, L.J., Zenilman, J. and Sears, C.L. 1995. Clinical manifestations and risk factors of *Pseudomonas aeruginosa* infection in patients with AIDS. Journal of Infectious Diseases 171(4): 930-937.

Drosinos, E.H. and Board, R.G. 1995. Microbial and physicochemical attributes of minced lamb: Sources of contamination with pseudomonads. Food Microbiology 12: 189-197.

Duffy, B.K. and Defago, G. 1995. Fungal inhibition and suppression of root disease using clinical isolates of *Pseudomonas aeruginosa*. Phytopathology 85: 1188.

Duijff, B.J., Baker, P.A.H.M. and Schippers, B. 1994. Suppression of Fusarium wilt of carnation by *Pseudomonas putida* WCS358 at different levels of disease incidence and iron availability. Biocontrol Science and Technology 4: 279-288.

Durbin, R.D. 1992. Role of toxins for plant-pathogenic pseudomonads. In: *Pseudomonas. Molecular Biology and Biotechnology* (pp. 43-55). American Society for Microbiology, Washington, D.C.

Eaton, R.W. and Timmis, K.N. 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. Journal of Bacteriology 168: 123-131.

Egusa, S. 1992. *Infectious Diseases of Fish*. A.A. Balkema Publishers, Bookfield, USA.

El Aalam, S., Pauss, A. and Lebeault, J. 1993. High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. Applied Microbiology and Biotechnology 39: 696-699.

El Said, H.M., Gewaily, E.M., Salem, S.H. and Tohamy, M.R. 1982. Biological and chemical control of *Pseudomonas aeruginosa*, the causal organism of blight disease of bean plants in Egypt. Egyptian Journal of Microbiology 17: 65-80.

Emori, T.G. and Gaynes, R.P. 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. Clinical Microbiology Reviews 6: 428-442.

England, L.S., Lee, H. and Trevors, J.T. 1993. Recombinant and wild-type *Pseudomonas aureofaciens* strains in soil: survival, respiratory activity and effects on nodulation of whitebean *Phaseolus vulgaris* L. by *Rhizobium* species. Molecular Ecology 2: 303-313.

Ensley, B.D. 1994. Designing pathways for environmental purposes. Current Opinion in Biotechnology 5: 247-248.

Faegri, A., Torsvik, V.L. and Goksoyr, J. 1977. Bacterial and fungal activities in soil: Separation of bacteria and fungi by a rapid fractionation centrifugation technique. Soil Biology and Biochemistry 9: 105-112.

Fava, F., Gioia, D.D. and Marchetti, L. 1993. Characterization of a pigment produced by *Pseudomonas fluorescens* during 3-chlorobenzoate co-metabolism. Chemosphere 27: 825-835.

Festl, H.W., Ludwig, W. and Schleifer, K.H. 1986. A DNA hybridization probe for the *Pseudomonas fluorescens* group. Applied and Environmental Microbiology 51: 1190-1194.

Fick, R.B., Jr. 1993. *Pseudomonas aeruginosa* - the microbial hyena and its role in disease: An introduction. In: Fica, R.B., Jr. (ed.). *Pseudomonas aeruginosa the opportunist: Pathogenesis and disease* (pp. 1-5). CRC Press, Boca Raton, Fla.

Fisher, A.A. 1994. Folliculitis from the use of a “loofah” cosmetic sponge [news]. Cutis 54: 12-13.

Fisher, M.C., Goldsmith, J.F. and Gilligan, P.H. 1985. Sneakers as a source of *Pseudomonas aeruginosa* in children with osteomyelitis following puncture wounds. Journal of Pediatrics 106: 607-609.

Flaishman, M., Eyal, Z., Voisard, C. and Haas, D. 1990. Suppression of *Septoria tritici* by phenazine or siderophore-deficient mutants of *Pseudomonas*. Current Microbiology 20: 121-124.

Ford, S.F. and Olsen, B. 1988. Methods for detecting genetically engineered microorganisms in the environment. Advances in Microbial Ecology 10: 45-79.

Foreman, N.K., Wang, W.C., Cullen, E.J., Stidham, G.L., Pearson, T.A. and Shenep, J.L. 1991. Endotoxic shock after transfusion of contaminated red blood cells in a child with sickle cell disease. Pediatric Infectious Disease Journal 10: 624-626.

Franzetti, F., Cernuschi, M., Esposito, R. and Moroni, M. 1992. *Pseudomonas* infections in patients with AIDS and AIDS-related complex. Journal of Internal Medicine 231(4): 437-443.

Fredrickson, J.K., Bentjen, S.A., Bolton, H. Jr. and Van voris, P. 1989. Fate of Tn*5* mutants of growth-inhibiting *Pseudomonas* sp. in intact soil-core microcosms. Canadian Journal of Microbiology 35: 867-873.

Freitas, De, J.R. and Germida, J.J. 1990. Plant growth promoting rhizobacteria for winter wheat. Canadian Journal of Microbiology 36: 265-272.

Freitas, De, J.R., Germida, J.J. and De Freitas, J.R. 1991. *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizoctonia solani*. Canadian Journal of Microbiology 37: 780-784.

Fujita, M., Ike, M., Hioki, J.I., Kataoka, K. and Takeo, M. 1995. Trichloroethylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. Journal of Fermentation and Bioengineering 79: 100-106.

Fukui, R., Schroth, M.N., Hendson, M., Hancock, J.G. and Firestone, M.K. 1994. Growth patterns and metabolic activity of *Pseudomonas* in sugar beet spermospheres: relationship to pericarp colonization by *Pythium ultimum*. Phytopathology 84(11): 1331-1338.

Gallagher, P.G. and Watanakunakorn, C. 1990. *Pseudomonas* bacteremia in a community teaching hospital, 1980-1984. Review of Infectious Diseases 11(6): 846-852.

Galli, E., Barbiere, P. and Bestetti, G. 1992. Potential of pseudomonads in the degradation of methylbenzenes. In: Galli, E., Silver, S. and Witholt, B. (eds). *Pseudomonas Molecular Biology and Biotechnology*. American Society of Microbiology.

Gamliel, A. and Katan, J. 1992. Chemotaxis of fluorescent pseudomonads towards seed exudates and germinating seeds in solarized soil. Phytopathology 82: 328-332.

Gamliel, A. and Katan, J. 1993. Suppression of major and minor pathogens by fluorescent pseudomonads in solarized and nonsolarized soils. Phytopathology 83: 68-75.

Gannon, J.T., Manial, V.B. and Alexander, M. 1991. Relationship between cell surface properties and transport of bacteria through the soil. Applied and Environmental Microbiology 57: 190-193.

Gatti, R. and Nigrelli, D. 1984. Haemorrhagic septicaemia in catfish. Pathogenicity of the strains isolated and reproducibility of the disease. Obbiettivi e Documenti Veterinari 5: 61-63.

Gaudet, D.A., Sands, D.C., Mathre, D.E. and Ditterline, R.L. 1980. The role of bacteria in the root and crown rot complex of irrigated sainfoin in Montana. Phytopathology 70: 161-167.

Genthner, F.J., Ross, S.S., Campbell, R.P. and Fournie, J.W. 1993. Fate and survival of microbial pest control agents in nontarget aquatic organisms. Diseases of Aquatic Organisms 16: 157-162.

Gilardi, G.L. 1991. *Pseudomonas* and related genera. In: Balows, A., Hausler, W.J., Herrmann, K.L., Isenberg, H.D. and Shadomy, H.D. (eds). *Manual of Clinical Microbiology* (fifth edition). American Society for Microbiology, Washington, D.C.

Gilligan, P.H. 1991. Microbiology of airway disease in patients with cystic fibrosis. Clinical Microbiology Reviews 4:35-51.

Glandorf, D.C.M., Peters, L.G.L., van der Sluis, I., Bakker, P.A.H.M. and Schippers, B. 1993. Crop specificity of rhizosphere pseudomonads and the involvement of root agglutinins. Soil Biology and Biochemistry 25: 981-989.

Glandorf, D.C.M., van der Sluis, I., Anderson, A.J., Bakker, P.A.H. and Schippers, B. 1994. Agglutination, adherence and root colonization by fluorescent pseudomonads. Applied and Environmental Microbiology 60: 1726-1733.

Glazebrook, J.S. and Campbell, R.S.F. 1990. A survey of the diseases of marine turtles in northern Australia. I. Farmed turtles. Diseases of Aquatic Organisms 9: 83-95.

Gonzalez, C.F., Layher, S.K., Vidaver, A.K. and Olsen, R.H. 1984. Transfer, mapping and cloning of *Pseudomonas syringae* pv. *syringae* plasmid pcg131 and assessment of its role in virulence. Phytopathology 74: 1245-50.

Gottlieb, T, Funnell, G. and Gosbell, I. 1991. *Pseudomonas fluorescens* pseudobacteraemia. Med. J. Aust. 155: 854-855.

Green, S.K., Schroth, M.N., Cho, J.J., Kominos, S.D. and Vitanza-Jack, V.B. 1974. Agricultural plants and soil as a reservoir for *Pseudomonas aeruginosa*. Applied Microbiology 28(6): 987-991.

Greer, G.G. 1989. Bacteria and meat quality. Canadian Institute of Food Science and Technology Journal 22(2): 116-117.

Grewal, P.S. 1991. Effects of *Caenorhabditis elegans* (Nematoda: Rhabditidae) on the spread of the bacterium *Pseudomonas tolaasii* in mushrooms *(Agaricus bisporus*). Annals of Applied Biology 118(1): 47-55.

Gross, D. 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. Journal of Applied Bacteriology 58: 167-174.

Gustafson, T.L., Band, L.D., Hutcheson, R.H. and Schaffer, W. 1983. *Pseudomonas folliculitis*: an outbreak and review. Review of Infectious Diseases 5:1-8.

Haefele, D.M. and Lindow, S.E. 1987. Flagella motility confers epiphytic fitness advantages upon *Pseudomonas syringae*. Applied and Environmental Microbiology 53: 2528-2533.

Hafez, H.M., Woernle, H. and Heil, G. 1987. *Pseudomonas aeruginosa* infections in turkey poults and treatment trials with apramycin. Berliner und Munchen Tierarztliche Wochenschrift 100(2): 48-51.

Hamdy, A.H.A., Farag, Y.A. and Micheal, F.F. 1974. *Pseudomonas aeruginosa* infection as a possible cause of infertility in Friesan bulls. Journal of the Egyptian Veterinary Medical Association 34: 612-622.

Handley, B.A. and Webster, A.J.F. 1993. Some factors affecting airborne survival of *Pseudomonas fluorescens* indoors. Journal of Applied Bacteriology 75: 35-42.

Haniotakis, G. and Avtizis, N. 1977. Mortality in *Dacus oleae* through infection with *Pseudomonas putida*. Ann. Zool. Ecol. Animal 9: 299-312.

Harrison, L.A., Letendre, L., Kovacevich, P., Pierson, E. and Weller, D. 1993. Purification of an antibiotic effective against *Gaeumannomyces graminis* var. *tritici* produced by a biocontrol agent, *Pseudomonas aureofaciens*. Soil Biology and Biochemistry 25(2): 215-221.

Hartel, P.G., Fuhrman, J.J., Johnson, W.F. and Lawrence, E.G. 1994. Survival of *lac* ZY-containing *Pseudomonas putida* strain under stressful abiotic soil conditions. Soil Science Society of America Journal 58: 770-776.

Hatai, K. and Willoughby, L.G. 1988. *Saprolegnia parasitica* from rainbow trout inhibited by the bacterium *Pseudomonas fluorescens*. Bulletin of the European Association of Fish Pathologists 8: 27-29.

Hatai, K., Egusa, S., Nakajima, M., and Chikahata, H. 1975. *Pseudomonas chlororaphis* as a fish pathogen. Bulletin of the Japanese Society of Scientific Fisheries 41(11): 1203.

Heijnen, C.E., Koh-A-Hin, C.H. and van Elsas, J.D. 1993. Root colonization by *Pseudomonas fluorescens* introduced into soil amended with bentonite. Soil Biology and Biochemistry 25: 239-246.

Hepperly, P.R. and Ramos-Davila, E. 1986. Bacterial basal rot of straw mushrooms. Journal of Agriculture of the University of Puerto Rico 70: 219-221.

Herald, P.J. and Zottola, E.A. 1989. Effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel. Journal of Food Science 54(2): 461-464.

Herron, P.R. and Wellington, E.M.H. 1990. New method for extraction of streptomycete spores from soil and application to the study of lysogeny in sterile amended and non sterile soil. Applied and Environmental Microbiology 56: 1406-1412.

Hickey, W.J. and Focht, D.D. 1992. Degradation of mono-, di-, and trihalogenated benzoic acids by *Pseudomonas aeruginosa* JB2. Applied and Environmental Microbiology 56: 3842-3850.

Hildebrand, D.C., Palleroni, N.J., Hendson, M., Toth, J. and Johnson, J.L. 1994. *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers. International Journal of Systematic Bacteriology 44: 410-415.

Hildebrand, P.D. 1989. Surfactant-like characteristics and identity of bacteria associated with broccoli head rot in Atlantic Canada. Canadian Journal of Plant Pathology 11: 205-214.

Hildebrand, P.D. 1994. Head rot. In: Howard, R.J., Garland, J.A. and Seaman, W.L. (eds). *Diseases and Pests of Vegetable Crops in Canada* (pp. 94-95). The Canadian Phytopathological Society and Entomological Society of Canada. Ottawa, Canada.

Hirano, S.S. and Upper, C.D. 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annual Review of Phytopathology 21:243-269.

Hirano, S.S. and Upper, C.D. 1990. Population biology and epidemiology of *Pseudomonas syringae*. Annual Review of Phytopathology 28: 155-177.

Hirano, S.S. and Upper, C.D. 1992. Population dynamics of *Pseudomonas syringae* in the phyllosphere. In: Galli, E., Silver, S. and Witholt, B. (eds). *Pseudomonas. Molecular Biology and Biotechnology*. American Society for Microbiology, Washington, DC.

Hirano, S.S. and Upper, C.D. 1993. Dynamics, spread, and persistence of a single genotype of *Pseudomonas syringae* relative to those of its conspecifics on populations of snap bean leaflets. Applied and Environmental Microbiology 59(4): 1082-1091.

Hiraishi, A., Morishima, Y. and Takeuchi, J.-I. 1991. Numerical analysis of lipoquinone patterns in monitoring bacterial community dynamics in wastewater treatment systems. Journal of General and Applied Microbiology 37: 57-70.

Holben, W.E., Jansson, J.K., Chelm, B.K. and Tiedje, J.M. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Applied and Environmental Microbiology 54: 703-711.

Holder, I.A. 1993. *Pseudomonas aeruginosa* virulence-associated factors and their role in burn wound infections. In: Fica, R.B. (ed.). *Pseudomonas aeruginosa the opportunist. Pathogenesis and disease* (pp. 235-245). CRC Press: Boca Raton.

Holland, S.P., Pulido, J.S., Shires, T.K. and Costerton, J.W. 1993. *Pseudomonas aeruginosa* ocular infections. In: Fica, R.B. (ed.). *Pseudomonas aeruginosa the opportunist. Pathogenesis and disease* (pp. 159-176). CRC Press: Boca Raton.

Holloway, B.W. and Morgan, A.F. 1986. Genome organisation in *Pseudomonas.* Annual Review of Microbiology 40: 79-105.

Holloway, B.W., Dharmsthiti, S., Johnson, C., Kearney, A., Krishnapillai, V., Morgan, A.F., Ratnaningsih, E., Saffrey, R., Sinclair, M., Strom, D. and Zhang, C. 1990a. Chromosome organisation in *Pseudomonas aeruginosa* and *Pseudomonas putida.* In: Silver, S., Chakabarty, A.M., Iglewski, B. and Kaplan S. *Pseudomonas*: *Biotransformation, Pathogenesis and Evolving Biotechnology*. American Society for Microbiology, Washington, D.C.

Holloway, B.W., Dharmsthiti, S., Krishnapillai, V., Morgan, A., Obeyesekere, V., Ratnaningsih, E., Sinclair, M., Strom, C. and Zhang, C. 1990b. Patterns of gene linkages in *Pseudomonas* species. In: *The Bacterial Chromosome (*pp.97-105). American Society for Microbiology, Washington, D.C.

Holloway, B.W., Romling, U. and Tummler, B. 1994. Genomic mapping of *Pseudomonas aeruginosa PAO*. Microbiology 140: 2907-2929.

Honich, M. 1972. Outbreak of *Pseudomonas aeruginosa* infection among pheasant chicks. Magyar Allatorvosok Lapja 27(6): 329-335.

Hood, S.K. and Zottola, E.A. 1995. Biofilms in food-processing. Food Control 6(1): 9-18.

Hopkins, D.W., Macnaughton, S.J. and O'Donnell, A.G. 1991. A dispersion and differential centrifugation technique for representatively sampling microorganisms from soil. Soil Biology and Biochemistry 23: 227-232.

Horohov, D.W. and Dunn, P.E. 1984. Role of hemocytotoxins in the pathogenicity of *Pseudomonas aeruginosa* in larvae of the tobacco hornworm, *Manduca sexta*. Journal of Invertebrate Pathology 43: 297-298.

Howard, R.J., Garland, J.A. and Seaman, W.L. 1994. *Diseases and Pests of Vegetable Crops in Canada: An Illustrated Compendium*. The Canadian Phytopathological Society and The Entomological Society of Canada. Ottawa, Canada.

Hungerford, T.G. 1990. *Diseases of Livestock* (9th Edition). McGraw-Hill Book Co., Toronto.

Hutchison, M.L. and Johnstone, K. 1993. Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. Physiological and Molecular Plant Pathology 42(5): 373-384.

Hwang, S.F., Howard, R.J. and Goatcher, L. 1989. Bacteria associated with crown and root rot of sainfoin in southern Alberta. Canadian Plant Disease Survey 69: 5-8.

Iglewski, B.H., Rust, L. and Bever, R.A. 1990. Molecular analysis of *Pseudomonas aeruginosa* elastase. In: Silver, S., Chakabarty, A.M., Iglewski, B. and Kaplan, S. (eds). *Pseudomonas: Biotransformation, Pathogenesis and Evolving Biotechnology.* American Society for Microbiology, Washington, D.C.

Imayasu, M., Petroll, W.M., Jester, J.V., Patel, S.K., Ohashi, J. and Cavanagh, J.D. 1994. The relation between contact lens oxygen transmissibility and binding of *Pseudomonas aeruginosa* to the cornea after overnight wear. Ophthalmology 101: 371-388.

Ishihara, S., Takino, M., Okada, Y. and Mimura, K. 1995. Septic shock due to *Pseudomonas aeruginosa* in a previously healthy woman. Intensive Care Medicine 21(3): 226-228.

Iwasaki, K., Uchiyama, H. and Yagi, O. 1993. Survival and impact of genetically engineered *Pseudomonas putida* harboring mercury resistance gene in aquatic microcosms. Biosci. Biotech. Biochem. 57: 1264-1269.

Iwasaki, K., Uchiyama, H. and Yagi, O. 1994. Survival and impact of genetically engineered *Pseudomonas putida* harboring mercury resistance gene in soil microcosms. Biosci. Biotech. Biochem. 58: 1264-1269.

Jacobson, E., Gaskin, J.M., Page, D., Iverson, W.O. and Johnson, J.W. 1981. Illness associated with paramyxo-like virus infection in a zoologic collection of snakes. Journal of the American Veterinary Medical Association 179(11): 1227-1230.

Jacoby, G.A. 1975. Properties of R plasmids in *Pseudomonas aeruginosa*. In: Schlessinger, D. (ed.). Microbiology 1974. American Society for Microbiology, Washington, D.C.

Jacques, M.A., Kinkel, L.L. and Morris, C.E. 1995. Population sizes, immigration, and growth of epiphytic bacteria on leaves of different ages and positions of field-grown endive (*Cichorium endivia* var latifolia). Applied and Environmental Microbiology 61(3): 899-906.

Jain, D.K., Lee, H. and Trevors, J.T. 1992. Effect of addition of *Pseudomonas aeruginosa* Ug2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. Journal of Industrial Microbiology 10(2): 87-93.

Jain, N., Srivastava, S.K. and Srivastava, A.K. 1993. Optimization of culture parameters for degradation of black liquor by *Pseudomonas putida*. Fresenius Environmental Bulletin 2: 705-710.

Jain, R.K., Burlage, R.S. and Sayler, G.S. 1988. Methods for detecting recombinant DNA in the environment. CRC Critical Reviews in Biotechnology 8: 33-84.

James, D.W., Jr., Suslow, T.V. and Steinback, K.E. 1985. Relationship between rapid, firm adhesion and long-term colonization of roots by bacteria. Applied and Environmental Microbiology 50: 392-397.

James, R.R. and Lighthart, B. 1992. The effect of temperature, diet, and larval instar on the susceptibility of an aphid predator, *Hippodamia convergens* (Coleoptera: Coccinellidae), to the weak bacterial pathogen *Pseudomonas fluorescens*. Journal of Invertebrate Pathology 60: 215-218.

Janiyani, K.L., Wate, S.R. and Joshi, S.R. 1993. Morphological and biochemical characteristics of bacterial isolates degrading crude oil. Journal of Environmental Science and Health - Part A - Environmental Science and Engineering 28: 1185-1204.

Janse, J.D., Derks, J.H.J., Spit, B.E. and van der Tuin, W.R. 1992. Classification of fluorescent soft rot *Pseudomonas* bacteria, including *P. marginalis* strains, using whole cell fatty acid analysis. Systematic and Applied Microbiology 15: 538-555.

Jarvis, J.G. and Skipper, J. 1994. *Pseudomonas* osteochondritis complicating puncture wounds in children. J. Pediatr. Orthop. 14(6): 755-759.

Jarvis, W.R. and Martone, W.J. 1992. Predominant pathogens in hospital infections. Journal of Antimicrobial Chemotherapy 29(suppl. A): 19-24.

Jay, J.M. 1992. *Modern Food Microbiology* (4th Edition). Chapman and Hall, New York.

Jensen, L.B., Ramos, J.L., Kaneva, Z. and Molin, S. 1993. A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli* gef gene. Applied and Environmental Microbiology 59: 3713-3717.

Johnson J.L. and Palleroni, N.J. 1989. Deoxyribonucleic acid similarities among *Pseudomonas* species. International Journal of Systematic Bacteriology 39: 230-235.

Johnson, K.B., Stockwell, V.O, Burgett, D.M., Sugar, D. and Loper, J.E. 1993. Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms. Phytopathology 83: 478-484.

Jones, R.A., Broder, M.W. and Stotsky, G. 1991. Effects of genetically engineered microorganisms on nitrogen transformations and nitrogen-transforming microbial populations in soil. Applied and Environmental Microbiology 57: 3212-3219.

Jones, R.N., Aldridge, K.E., Allen, S.D., Barry, A.L., Fuchs, P.C., Gerlach, E.H. and Pfaller, M.A. 1989. Multicenter *in vitro* evaluation of Sm-7338, a new carbapenem. Antimicrob. Agents Chemother. 33: 562-565.

Kenna, M.A. 1994. Treatment of chronic suppurative otitis media. Otolaryngol. Clin. North Am. 27: 457-472.

Khalid, A.M. and Aleem, M.I.H. 1991. Application of microorganisms in coal cleaning process. In: Proceedings of an International Symposium on Biotechnology for Energy, Dec. 16-21, 1989 (pp.379-403). Faisalabad, Pakistan.

Khanna, M. and Stotzky, G. 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. Applied and Environmental Microbiology 58: 1930-1939.

Khanna, P. and Olivier, J. M. 1989. Rapid screening of antagonists of blotch bacterium, *Pseudomonas tolaasii*. Indian Phytopathology 42(1): 139-141.

Kidambi, S.P., Ripp, S. and Miller, R.V. 1994. Evidence for phage-mediated gene transfer among *Pseudomonas aeruginosa* strains on the phylloplane. Applied and Environmental Microbiology 60: 496-500.

Kimura, T., Yoshimizu, M., Ezura, Y. and Kamei, Y. 1990. An antiviral agent (46NW-04A) produced by *Pseudomonas* sp. and its activity against fish viruses. Journal of Aquatic Animal Health 2: 12-20.

King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. Journal of Laboratory and Clinical Medicine 44: 301-307.

King, J., Digrazia, P., Applegate, B., Burtage, R., Sanseverino, J., Dunbar, P., Larimer, F. and Sayler, G. 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science 249: 778-780.

Kinkel, L.L. and Lindow, S.E. 1993. Invasion and exclusion among coexisting *Pseudomonas syringae* strains on leaves. Applied and Environmental Microbiology 59(10): 3447-3454.

Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H. and Takizawa, N. 1994. Cloning and characterization of a chromosomal gene cluster, pah, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. Journal of Bacteriology 176: 2439-2443.

Kloepper, J.W., Hume, D.J., Scher, F.M., Singleton, C., Tipping, B., Laliberte, M., Frauley, K., Kutchaw, T., Simonson, C., Lifshitz, R., Zaleska, I. and Lee, L. 1988. Plant growth promoting rhizobacteria on canola (rapeseed). Plant Disease 72: 42-46.

Kluepfel, D.A. and Tonkyn, D.W. 1991a. Release of soil-borne genetically modified bacteria: biosafety implications from contained experiments. McKenzie, D.R. and Henry, S.C. (eds). *Proceedings of the International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*, Kiawah Island, South Carolina, November 27, 1990 (pp. 55-65). Bethesda, Maryland: Agricultural Research Institute.

Kluepfel, D.A., Kline, E.L., Hughes, T., Skipper, H., Gooden, D., Drahos, D.J., Barry, G.F., Hemming, B.C. and Brandt, E.J. 1991b. Field testing of a genetically engineered rhizosphere inhabiting pseudomonad: development of a model system. In: McKenzie, D.R. and Henry, S.C. (eds). *Proceedings of the International Symposium on the Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms*, Kiawah Island, South Carolina, November 27, 1990 (pp. 189-199). Bethesda, Maryland: Agricultural Research Institute.

Kluepfel, D.A., Kline, E.L., Skipper, H.D., Hughes, T.A., Gooden, D.T., Drahos, D.J., Barry, G.F., Hemming, B.C. and Brandt, E.J. 1991c. The release and tracking of genetically engineered bacteria in the environment. Phytopathology 81(3) 348-352.

Knudsen, G.R. 1989. Model to predict aerial dispersal of bacteria during environmental release. Applied and Environmental Microbiology 55: 2641-2647.

Korfhagen, T.R., Sutton, L. and Jacoby, G.A. 1978. Classification and physical properties of *Pseudomonas* plasmids. In: Sclessinger, D. (ed.). Microbiology 1978, pp. 221-224. American Society for Microbiology, Washington, D.C.

Kropec, A., Lemmen, S., Wursthorn, M. and Daschner, F.D. 1994. Combination effect of meropenem with aminoglycosides and teicoplannin on *Pseudomonas* and enterococci. Infection 22(4): 306-308.

Kudoh, I., Wiener-Kronish, J.P., Hashimoto, S., Pittet, J.F. and Frank, D. 1994. Exoproduct secretions of *Pseudomonas aeruginosa* strains influence severity of alveolar epithelial injury. American Journal of Physiology 267: 551-556.

Kunz, D., Nagappan, O., Silva-Avalos, J. and DeLong, G. 1992. Utilization f-cyanide as a nitrogenous substrate by *Pseudomonas fluorescens NCIMB11764*: evidence for multiple pathways of metabolic conversion. Applied and Environmental Microbiology 58: 2022-2029.

Kusuda, R. and Toyoshima, T. 1976. Properties of pathogenic *Pseudomonas* isolated from cultivated yellowtail. Fish Pathology 11: 133-139.

Kusuda, R., Toyoshima, T. and Nishioka, J. 1974. Properties of pathogenic *Pseudomonas* isolated from cultivated *Evynnis japonica*. Fish Pathology 9: 71-78.

Ladds, P.W., Thomas, A.D., Speare, R. and Brown, A.S. 1990. Melioidosis in a koala. Australian Veterinary Journal 67: 304-305.

Lajoie, C.A., Layton, A.C. and Sayler, G.S. 1994. Cometabolic oxidation of polychlorinated biphenyls in soil with a surfactant-based field application vector. Applied and Environmental Microbiology 60: 2826-2833.

Lambert, B., Joos, H., Dierickx, S., Vantomme, R., Swings, J., Kersters, K. and van Montagu, M. 1990. The identification and plant interaction of a *Phyllobacterium* sp. a predominant rhizobacterium of young sugar beet plants. Applied and Environmental Microbiology 56: 1093-1102.

Lavery, L.A., Harkless, L.B., Felder-Johnson, K. and Mundine, S. 1994. Bacterial pathogens in infected puncture wounds in adults with diabetes. Journal of Foot and Ankle Surgery 33: 91-97.

Lebeda, A., Kudela, V. and Jedlickova, Z. 1984. Pathogenicity of *Pseudomonas aeruginosa* for plants and animals. Acta Phytopathologica Academiae Scientiarum Hungaricae 19: 271-284.

Lee, Y.H., Shim, G.Y., Lee, E.J. and Mew, T.W. 1990. Evaluation of biocontrol activity of fluorescent pseudomonads against some rice fungal diseases in vitro and greenhouse. Korean Journal of Plant Pathology 6(1): 73-80.

Lee, J-Y., Jung, K-H., Choi, S.H. and Kim, H-S. 1995. Combination of the *tod* and *tol* pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralization of a benzene, toluene, and *p*-xylene mixture. Applied and Environmental Microbiology 61: 2211-2217.

Legard, D.E., McQuilken, M.P., Whipps, J.M., Fenlon, J.S., Fermor, T.R., Thompson, I.P., Bailey, M.J. and Lynch, J.M. 1994. Studies of seasonal changes in the microbial populations on the phyllosphere of spring wheat as a prelude to the release of a genetically modified microorganism. Agriculture, Ecosystems and Environment 50(2): 87-101.

Legent, F., Bordure, P., Beauvillain, C. and Berche, P. 1994. Controlled prospective study of oral ciprofloxacin versus amyoxicillin/clavulanic acid in chronic suppurative otitis media in adults. Chemotherapy 40 (suppl 1): 16-23.

Lejeune, P. and Mergeay, M. 1980. R plasmid-mediated chromosome mobilization in *Pseudomonas fluorescens* G2. Arch. Physiol. Biochim. 80: 289-290.

Leser, T. 1995. Journal of Microbiological Methods 22: 249-262.

Lessie, T.G, Wood, M.S., Bryne, A. and Ferrante, A. 1990. Transposable gene-activating elements in *Pseudomonas cepacia*. In: Silver, S., Chakabarty, A.M., Iglewski, B. and Kaplan, S. (eds). *Pseudomonas: Biotransformation, Pathogenesis and Evolving Biotechnology*. (pp. 279-291). American Society for Microbiology, Washington, D.C.

Li, M.F. and Flemming, C. 1967. A proteolytic pseudomonad from skin lesions of rainbow trout (*Salmo gairdneri*). I. Characteristics of the pathogenic effects and the extracellular proteinase. Canadian Journal of Microbiology 13: 405-416.

Liao, C.H. 1989. Antagonism of *Pseudomonas putida* strain PP22 to phytopathogenic bacteria and its potential use as a biocontrol agent. Plant disease 73: 223-226.

Liao, C.H. 1991. Cloning of pectate lysase gene pel from *Pseudomonas fluorescens* and detection of sequences homologous to pel in *Pseudomonas viridiflava* and *Pseudomonas putida*. Journal of Bacteriology 173: 4386-4393.

Lilley, A.K., Fry, J.C., Day, M.J. and Bailey, M.J. 1994. *In situ* transfer of an exogenously isolated plasmid between *Pseudomonas* spp. in sugar beet rhizosphere. Microbiology 140: 27-33.

Lin, M.Y., Cheng, M.C., Huang, K.J. and Tsai, W.C. 1993. Classification, pathogenicity, and drug susceptibility of hemolytic gram-negative bacteria isolated from sick or dead chickens. Avian Diseases 37: 6-9.

Lindemann, J. and Upper, C.D. 1985. Aerial dispersal of epiphytic bacteria over bean plants. Applied and Environmental Microbiology 50: 1229-1232.

Lindemann, J., Constantinidou, H.A., Barchet, W.R. and Upper, C.D. 1982. Plants as sources of airborne bacteria including ice nucleation-active bacteria. Applied and Environmental Microbiology 44: 1059-1063.

Lindow, S.E. 1983. The role of bacterial ice nucleation in frost injury to plants. Annual Review of Phytopathology 21: 363-384.

Lindow, S.E. 1992. Ice- strains of *Pseudomonas syringae* introduced to control ice nucleation active strains on potato. In: Tjamos, E.S., Papavizas, G.C. and Cook, R.J. (eds). *Biological Control of Plant Diseases*. Plenum Press, New York.

Lindow, S.E. and Panopoulos, N.J. 1988. Field tests of recombinant Ice- *Pseudomonas syringae* for biological frost control in potato. In: Sussman, M., Colins, C.H. and Skinner, F.A. (eds). *Proceedings for the First International Conference on Release of Genetically Engineered Microorganisms.* Academic Press, London.

Lindow, S.E., Knudsen, G.R., Seidler, R.J., Walter, M.V., Lambou, V.W., Amy, P.S., Schmedding, D., Prince, V. and Hern, S. 1988. Aerial dispersal and epiphytic survival of *Pseudomonas syringae* during a pretest for the release of genetically engineered strains into the environment. Applied and Enviornmental Microbiology 54(6): 1557-1563.

Lorenz, M.G. and Wackernagel, W. 1991. High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorous source. Applied and Environmental Microbiology 57: 1246-1251.

Lorenz, M.G., Aardema, B.W. and Wackernagel, W. 1988. Highly efficient genetic transformation of *Bacillus subtilis* attached to sand grains. Journal of General Microbiology 134: 107-112.

Lucht, R.F., Fresard, A., Berthelot, P., Farizon, F., Aubert, Dorche, G. and Bousquet, G. 1994. Prolonged treatment of chronic *Pseudomonas aeruginosa* osteomyelitis with a combination of two effective antibiotics. Infection 22: 276-280.

Lynch, J.M. 1990. Longevity of bacteria: considerations in environmental release. Current Microbiology 20: 387-389.

Lysenko, O. 1974. Bacterial exoenzymes toxic for insects: proteinase and lecithinase. Journal of Hygiene, Epidemiology, Microbiology and Immunology 18(3): 347-352.

MacDonald, R.M. 1986. Sampling soil microfloras: dispersion of soil by ion exchange and extraction of specific microorganisms from suspension by elutriation. Soil Biology and Biochemistry 18: 399-406.

MacFarlane, L., Oppenheim, B.A. and Lorrigan, P. 1991. Septicaemia and septic arthritis due to *Pseudomonas putida* in a neutropenic patient. Journal of Infection 23(3): 346-347.

Macnaughton, S.J., Rose, D.A. and O'Donnell, A.G. 1992. Growth and survival of genetically engineered *Pseudomonas putida* in soils of varying texture. Journal of General Microbiology 138: 667-673.

Madhavan, T., Fisher, E.J., Cox, F. and Quinn, E.J. 1973. *Pseudomonas putida* and septic arthritis. Annals of Internal Medicine 78: 971-972.

Madsen, E.L. and Alexander, M. 1982. Transport of *Rhizobium* and *Pseudomonas* through soil. Soil Science Society of America Journal 46: 557-560.

Maharaj, R., Zielinski, N.A. and Chakrabarty, A.M. 1992. Environmental regulation of alginate gene expression in *Pseudomonas aeruginosa*. In: *Pseudomonas.* Molecular Biology and Biotechnology (pp. 105-115). American Society for Microbiology, Washington, D.C.

Mallolas, J.H., Gatell, J.M., Miro, J.M., Marco, F. and Soriano, E. 1990. Epidemiologic characteristics and factors influencing the outcome of *Pseudomonas aeruginosa* bacteremia. Reviews of Infectious Disease 12: 718-719.

Mancini, P., Fertels, S., Nave, D. and Gealt, M. 1987. Mobilization of plasmid pHSV106 from *Escherichia coli* HB101 in a laboratory-scale waste treatment facility. Applied and Environmental Microbiology 53: 665-671.

Manohar, L., Sheony, M. G., Reddy, T. K. K. and Chadramohan, K. 1976. A new bacterial fish pathogen causing skin disease in catfish *Clarias batrachus* Linn. (*Pseudomonas aeruginosa*). Current Research, University of Agricultural Sciences, Bangalore, India 5(5): 76-77.

Mathre, D. E., Callan, N. W., Johnston, R. H., Miller, J. B. and Schwend, A. 1994. Factors influencing the control of *Pythium ultimum*-induced seed decay by seed treatment with *Pseudomonas aureofaciens* AB254. Crop Protection 13(4): 301-307.

Mazzola, M., Cook, R.J., Thomashow, L.S., Weller, D.M. and Pierson, L.S. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Applied and Environmental Microbiology 58(8): 2616-2624.

McCartney, H.A. and Butterworth, J. 1992. Effects of humidity on the dispersal of *Pseudomonas syringae* from leaves by water splash. Microbial Releases 1: 187-190.

McCubbin, M. and Fick, R.B. 1993. Pathogenesis of *Pseudomonas* lung disease in cystic fibrosis. In: Fica, R.B. (ed.) *Pseudomonas aeruginosa the opportunist. Pathogenesis and disease* (pp. 189-211). CRC Press: Boca Raton.

Membre, J.M. and Burlot, P.M. 1994. Effects of temperature, pH, and NaCl on growth and pectinolytic activity of *Pseudomonas marginalis*. Applied and Environmental Microbiology 60: 2017-2022.

Mergeay, M., Lejeune, P., Dadouk, A., Gerits, A.J. and Fabry, L. 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. 209: 61-70.

Miake, S., Nomoto, K., Yokokura, T., Yoshikai, Y., Mutai, M., and Nomoto, K. 1985. Protective effect of *Lactobacillus casei* on *Pseudomonas aeruginosa* infection in mice. Infection and immunity 48(2): 480-485.

Miller, H.J., Liljeroth, E., Henken, G. and van Veen, J.A. 1990b. Fluctuations in the fluorescent pseudomonad and actinomycete populations of rhizosphere and rhizoplane during the growth of spring wheat. Canadian Journal of Microbiology 36: 254-258.

Miller, R.V., Kokjohn, T.A. and Sayler, G.S. 1990a. Environmental and molecular characterization of systems which affect genome alteration in *Pseudomonas aeruginosa.* In*: Pseudomonas:* transformations, pathogenesis and evolving biotechnology. American Society for Microbiology, Washington., D.C.

Milus, E.A. and Rothrock, C.S. 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. Canadian Journal of Microbiology 39: 335-341.

Miyashita, T. 1984. *Pseudomonas fluorescens* and *Edwardsiella tarda* isolated from diseased tilapia. Fish Pathology 19: 45-50.

Miyazaki, T., Kubota, S.S. and Miyashita, T. 1984. A histopathological study of *Pseudomonas fluorescens* infection in tilapia. Fish Pathology 19: 161-166.

Moayyedi, P., Lynch, D. and Axon, A. 1994. *Pseudomonas* and endoscopy. Endoscopy 26(6): 554-558.

Molin, G. and Ternström, A. 1986. Phenotypically based taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water and soil. International Journal of Systematic Bacteriology 36: 257-274.

Molin, S., Boe, L., Jensen, L.B., Kristensen, C.S., Givskov, M., Ramos, J.L. and Bej, A.K. 1993. Suicidal genetic elements and their use in biological containment of bacteria. Annual Review of Microbiology 47: 139-166.

Morgan, J.A.W., Winstanley, C., Pickup, R.W. and Sanders, J.R. 1991. The rapid immunocapture of *Pseudomonas putida* cells from lake water using bacterial flagella. Applied and Environmental Microbiology 57: 503-509.

Morgan, J.A.W., Winstanley, C., Pickup, R.W., Jones, J.G. and Saunders, J.R. 1989. Direct phenotypic and genotypic detection of a recombinant pseudomonad population in lake water. Applied and Environmental Microbiology 55: 2537-2544.

Morrison, W.D., Miller, R.V. and Sayler, G.S. 1979. Frequency of F116-mediated transduction of *Pseudomonas aeruginosa* in a fresh water environment. Applied and Environmental Microbiology 36: 724-730.

Moulton, P.J., Vivian, A., Hunter, P.J. and Taylor, J.D. 1993. Changes in cultivar-specificity toward pea can result from transfer of plasmid RP4 and other incompatibility group P1 replicons to *Pseudomonas syringae* pv. *pisi*. Journal of General Microbiology 139: 3149-3155.

Munjal, A., Khanna, P.K. and Garcha, H. S. 1989. *In vitro* chemical and biological control of bacterial blotch of *Agaricus bisporus* (Sing.) In: Lange, K. Grabbe, and Hilber, O. (eds). *Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi*, Braunschweig, Germany, September 1987 (pp. 667-677). Mushroom Science XII, Part II. Braunschweig, Germany: International Society for Mushroom Science.

Murayama, A. and Saburo, T. 1970. Fragin, a new biologically active metabolite of *Pseudomonas*. Part III. Synthesis. Agricultural and Biological Chemistry 34: 122-130.

Murty, M.G., Srinivas, G. and Sekar, V. 1994. Production of a mosquitocidal exotoxin by a *Pseudomonas fluorescens* strain. Journal of Invertebrate Pathology 64: 68-70.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695-700.

Nair, N.G. and Fahy, P.C. 1972. Bacteria antagonistic to *Pseudomonas tolaasii* and their control of brown blotch of the cultivated mushroom *Agaricus bisporus*. Journal of Applied Bacteriology 35(3): 439-442.

Nashif, S.A. and Nelson F.E. 1953. The lipase of *Pseudomonas fragi*. II. Factors affecting lipase production. Journal of Dairy Science 36: 471-480.

Nemecek-Marshall, M., Laduca, R. and Fall, R. 1993. High-level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low-temperature. Journal of Bacteriology 175(13): 4062-4070.

Nensey, Y.M., Malhotra, S.K., Schubert, T.T. and Zonca, M.S. 1990. *Pseudomonas aeruginosa* bacteremia associated with sphincter of Oddi manometry. American Journal of Gastroenterology 85(4): 478-479.

Neu, H.C. 1985. Ecology, clinical significance and antimicrobial susceptibility of *Pseudomonas aeruginosa*. In: Gilardi, G.L. (ed.). *Nonfermentative gram-negative rods. Laboratory identification and clinical aspects* (pp. 117-158). Marcel Dekker Inc., New York.

Newbound, G.C., Speare, D.J., Hammell, K.L., Kent, M.L., Ostland, V.E. and Traxler, G.S. 1993. Chehalis River disease: a unique gill disease of salmonids. Canadian Journal of Fisheries and Aquatic Sciences 50: 1092-1100.

Nicas, T.I. and Iglewski, B.H. 1986. Toxins and virulence factors of *Pseudomonas aeruginosa*. In: Sokatch, J.R. (ed.), *The Biology of Pseudomonas* (pp. 195-213), The bacteria, Volume X. Academic Press, Toronto.

Noble, W.C. 1993. Gram-negative bacterial skin infections. Semin. Dermatol. 12: 336-341.

O'Morchoe, S.B., Ogaunseitan, O., Sayler, G.S. and Miller, R.V. 1988. Conjugal transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* strains in a freshwater environment. Applied and Environmental Microbiology 54: 1923-1929.

O'Sullivan, D.J. and O'Gara, F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews 56: 662-676.

Obukowicz, M. and Shaw, P.D. 1983. Tn*3* labeling of a cryptic plasmid found in the plant pathogenic bacterium *Pseudomonas tabaci* and mobilization of RSF1010 by donation. Journal of Bacteriology 155: 438-42.

Obukowicz, M. and Shaw, P.D. 1985. Construction of Tn*3*-containing plasmids from plant-pathogenic pseudomonads and an examination of their biological properties. Applied and Environmental Microbiology 49: 468-73.

OECD 1986. Recombinant DNA Safety Considerations. OECD, Paris, 1986.

OECD 1994a. Ottawa '92: The OECD Workshop on Methods for Monitoring Organisms in the Environment, Environment Monograph No. 90. OECD, Paris, 1994.

OECD 1994b. Compendium of Methods for Monitoring Organisms in the Environment, Environment Monograph No. 91. OECD, Paris, 1994.

OECD 1995. Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology, Environment Monograph No. 100. OECD, Paris, 1995.

OECD 1996. Industrial Products of Modern Biotechnology Intended for Release to the Environment, Environment Monograph No. 117. OECD, Paris, 1996.

Okaeme, A.N. 1989. Bacteria associated with mortality in tilapias, *Heterobranchus bidorsalis*, and *Clarias lazera*, in indoor hatcheries and outdoor ponds. Journal of Aquaculture in the Tropics 4: 143-146.

Omar, S.A. and Abd-Alla, M.H. 1994. Enhancement of faba bean nodulation, nitrogen fixation and growth by different microorganisms. *Biologia Plantarum* 36: 295-300.

Ononiwu, J.C. 1980. Transmissable enteritis of turkeys (Bluecomb) in Nigeria. *Bulletin of Animal Health and Protection in Africa* 28: 352-354.

Ormrod, D.J. and Jarvis, W.R. 1994. *Pseudomonas* diseases. In: Howard, R.J., Garland, J.A. and Seaman, W.L. (eds). *Diseases and Pests of Vegetable Crops in Canada* (pp. 146-147). The Canadian Phytopathological Society and Entomological Society of Canada. Ottawa, Canada.

Oyaizu, H. and Komgata, K. 1983. Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existance of 3-hydroxy fatty acids. Jouurnal of General and Applied Microbiology 29: 17-40.

Ozaktan, H. and Bora, T. 1994. Studies on identification of bacterial microflora of mushroom in Turkey. Journal of Turkish Phytopathology 23: 73-78.

Palleroni, N.J. 1981. Introduction to the family *Pseudomonadaceae*. In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schegel, H.G. (eds). *The Prokaryotes - A Handbook on Habitats, Isolation and Identification of Bacteria (*pp. 655-665), Chapter 59. Springer-Verlag, Berlin.

Palleroni, N.J. 1984. *Pseudomonadaceae.* In: Kreig, N.R. and Holt, J.G. (eds). *Bergey's Manual of Systematic Bacteriology* (pp. 141-219), Vol. 1. The Williams and Wilkins Co., Baltimore.

Palleroni, N.J. 1992a. Human and animal pathogenic pseudomonads. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. (eds). *The Prokaryotes,* Chapter 161 (pp. 3086-3103) (2nd Edition), Vol. III. Springer-Verlag, New York.

Palleroni, N.J. 1992b. Present situation of the taxonomy of the aerobic pseudomonads In: Galli, E., Silver, S. and Witholt, B. (eds). *Pseudomonas. Molecular Biology and Biotechnology (*pp. 105-115). American Society of Microbiology, Washington, D.C.

Palleroni, N.J. 1992c. *Pseudomonas* classification. Antonie v. Leeuwenhoek 64: 231-251.

Palleroni, N.J., Kunisawa, R., Contopoulou, R. and Doudoroff, M. 1973. Nucleic acid homologies in the genus *Pseudomonas*. International Journal of Systematic Bacteriology 23: 333-339.

Papadopoulou-Karabela, K., Iliadis, N., Liakos, V. and Bourdzy-Hatzopoulou, E. 1992. Experimental infection of honeybees by *Pseudomonas aeruginosa*. Apidologie 23(5): 393-397.

Papapetropoulou, M., Rodopoulou, G., Giannoulaki, E. and Stergiopoulos, P. 1994. Effect of temperature on antimicrobial susceptibilities of *Pseudomonas* species isolated from drinking water. Journal of Chemotherapy 6(6): 404-407.

Paul, J.H., Jeffrey, W.H. and Deflaun, M.E. 1987. Dynamics of extracellular DNA in the marine environment. Applied and Environmental Microbiology 53: 170-179.

Petrinec, Z., Naglic, T., Matasin Z. and Fijan, N. 1985. *Pseudomonas fluorescens* septicaemia in bighead (*Aristichthys nobilis* Rich) following handling. Veterinarski Arhiv. 55: 277-284.

Pierson, L.S. and Thomashow, L.S. 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Molecular Plant Microbe Interactions 5(4): 330-339.

Pillai, S.D., Josephson, K.L., Bailey, R.L., Gerba, C.P. and Pepper, I.L. 1991. Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. Applied and Environmental Microbiology 57: 2283-2286.

Pitt, T.L. 1990. *Pseudomonas*. In: Wilson, G., Miles, A. and Parker, M.T. (eds). *Topley and Wilson's Principles of Bacteriology, Virology and Immunity (*8th Edition). Edward Arnold, London.

Piwowarski, J.M. and Shaw, P.D. 1982. Characterization of plasmids from plant pathogenic pseudomonads. Plasmid 7: 85-94.

Plotkowski, M.C., Saliba, A.M., Pereira, S.H., Cervante, M.P. and Bajolet-Laudinat, O. 1994. *Pseudomonas aeruginosa* selective adherence to and entry into human epithelial cells. Infection and Immunity 62: 5456-5463.

Pollack, M. 1992. *Pseudomonas*. In: Gorbach, S.L., Bartlett, J.G. and Blackhew, N.R. (eds). *Infectious Diseases* (pp. 1502-1513). W.B. Saunders Co., Philadelphia/London.

Premalatha, A. and Rajakumar, G. S. 1994. Pentachlorophenol degradation by *Pseudomonas aeruginosa*. World Journal of Microbiology and Biotechnology 10: 334-337.

Prieto, M., Garciaarmesto, M.R., Garcialopez, M.L., Alonso, C. and Otero, A. 1992. Species of *Pseudomonas* obtained at 7° C and 30° C during aerobic storage of lamb carcasses. Journal of Applied Bacteriology 73(4): 317-323.

Prince, A. 1992. Adhesins and receptors of *Pseudomonas aeruginosa* associated with infection of the respiratory tract. Microbial Pathology 13: 251-260.

Prosser, J.I. 1994. Molecular marker systems for detection of genetically engineered microorganisms in the environment. Microbiology 140: 5-17.

Puckett, A., Davison, G., Entwistle, C.C. and Barbara, J.A.J. 1992. Post-transfusion septicaemia 1980-1989: importance of donor arm cleansing. Journal of Clinical Pathology 45: 155-157.

Pujar, B.G. and Ribbons, D.W. 1985. Phthalate metabolism in *Pseudomonas fluorescens* PHK: Purification and properties of 4,5-dihydroxyphthalate decarboxylase. Applied and Environmental Microbiology 49: 374-376.

Quant, R.L. and Mills, D. 1984. An integrative plasmid and multiple-sized plasmids of *Pseudomonas syringae* pv. *phaseolicola* have extensive homology. Molecular and General Genetics 193: 459-466.

Quinn, J.P. 1992. Intrinsic antibiotic resistance in *Pseudomonas aeruginosa*. In: Galli, E., Silver, S. and Witholt, B. (eds). *Pseudomonas. Molecular Biology and Biotechnology*. American Society for Microbiology, Washington, D.C.

Raaska, L. and Maffila-Sandholm, T. 1991. The antogonistic activity of *Pseudomonas chlororaphis* and *Pseudomonas fluorescens* against shiitake (*Lentinula edodes*) mycelia. Material und Organismen 26: 287-302.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shoa, J., Tompkins, R.G. and Ausubel, F.M. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science 268: 1899-1902.

Rainey, P.B., Brodey, C.L. and Johnstone, K. 1991. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. Physiological and Molecular Plant Pathology 39(1): 57-70.

Rainey, P.B., Brodey, C.L. and Johnstone, K. 1993. Identification of a gene cluster encoding three high-molecular-weight proteins, which is required for synthesis of tolaasin by the mushroom pathogen *Pseudomonas tolaasii*. Molecular Microbiology 8(4): 643-652.

Ramos, J.L., Duque, E. and Ramos-Gonzalez, M.I. 1991. Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. Applied and Environmental Microbiology 57: 260-266.

Ramos, J.L., Diaz, E., Dowling, D., de Lorenzo, V., Molin, S., O'Gara, F., Ramos, C. and Timmis, K.N. 1994. Behavior of designed bacteria for biodegradation. Bio/Technology 12: 1349-1356.

Ramos-Gonzalez, M.I., Duque, E. and Ramos, J.L. 1991. Conjugational transfer of recombinant DNA in cultures and in soils: Host range of *Pseudomonas putida* TOL plasmids. Applied and Environmental Microbiology 57: 3020-3027.

Ramos-Gonzalez, M.I., Ruiz-Cabello, F., Brettar, I., Garrido, F. and Ramos, J.L. 1992. Tracking genetically engineered bacteria: monoclonal antibodies against surface determinants of the soil bacterium *Pseudomonas putida* 2440. Journal of Bacteriology 174: 2978-2985.

Ramos-Gonzalez, M.I., Ramos-Diaz, M.A., Ramos, J.L. 1994. Chromosomal gene capture mediated by the *Pseudomonas putida* TOL catabolic plasmid. Journal of Bacteriology 174: 2978-2985.

Ratnaningsih, E., Dharmsthiti, S., Krishnapillai, A., Morgan, M., Sinclair, M. and Holloway, B.W. 1990. A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. Journal of General Microbiology 136: 2351-2357.

Rattray, E.A.S., Tyrrell, J.A., Prosser, J.I., Glover, L.A. and Killham, K. 1993. Effect of soil bulk density and temperature on wheat rhizosphere colonization by lux-marked *Pseudomonas fluorescens*. European Journal of Soil Biology 29: 73-82.

Richardson, P.N. 1993. Stipe necrosis of cultivated mushrooms (*Agaricus bisporus*) associated with a fluorescent pseudomonad. Plant Pathology 42: 927-929.

Richet, H., Escande, M.C., Marie, J.P., Zittoun, R. and Lagrange, P.H. 1989. Epidemic *Pseudomonas aeruginosa* serotype O16 bacteremia in hematology-oncology patients. Journal of Clinical Microbiology 27(9): 1992-1996.

Riley, M. 1989. Constancy and change in bacterial genomes. In: Poindexter, J.S. and Leadbeater, E.R. (eds). *Bacteria in Nature* (pp. 359-388).

Roberts, R.J. and Horne, M.T. 1978. Bacterial meningitis in farmed rainbow trout, *Salmo gairdneri* Richardson, affected with chronic pancreatic necrosis. Journal of Fish Diseases 1: 157-164.

Roilides, E., Butler, K.M., Husson, R.N., Mueller, B.U., Lewis, L.L. and Pizzo, P.A. 1992. *Pseudomonas* infections in children with human immunodeficiency virus infection. Pediatric Infectious Disease Journal 11(7): 547-553.

Romanowski, G., Lorenz, M.G. and Wackernagel, W. 1991. Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Applied and Environmental Microbiology 57: 1057-1061.

Romling, U., Grothues, D., Bautsch, W. and Tummler, B. 1989. A physical genome map of *Pseudomonas aeruginosa* PAO. EMBO Journal 8: 4081-4089.

Romling, U., Fiedler, B., Bosshammer, J., Grothues, D., Greipel, J., von der Hardt, J. and Tummler, B. 1994. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. Journal of Infectious Diseases 170: 1616-1621.

Romling, U., Wingender, J., Muller, H. and Tummler, B. 1994. A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. Applied and Environmental Microbiology 60: 1734-1738.

Ronchel, M.C., Ramos, C., Jensen, L.B., Molin, S. and Ramos, J.L. 1995. Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. Applied and Environmental Microbiology 61: 2990-2994.

Ros, S.P. 1989. *Pseudomonas* bacteremia in a previously healthy 2-year-old boy. Clinical Pediatrics (Phila.) 28(7): 336.

Rosak, D.B. and Colwell, R.R. 1987. Survival strategies of bacteria in the natural environment. Microbiological Reviews 51: 365-379.

Safwat, E.E.A., Awaad, M.H., Ammer, A.M. and El Kinawy, A.A. 1986. Studies on *Pseudomonas aeruginosa*, *Proteus vulgari*s and *Salmonella typhimurium* infection in ducklings. Egyptian Journal of Animal Protection 24: 287-294.

Sarsola, P., Taylor, D.J., Love, S. and McKellar, Q.A. 1992. Secondary bacterial infections following an outbreak of equine influenza. Veterinary Record 131: 441-442.

Sato, M., Nishiyama, K. and Shirata, A. 1983. Involvement of plasmid DNA in the productivity of coranatine by *Pseudomonas syringae* pv. *atropurpurea*. Annual Phytopathology Society, Japan 49: 522-528.

Sato, Y. et. al. 1986. *Pseudomonas aeruginosa* infection with granulomas in a Japanese ptarmigan. Journal of the Japan Veterinary Medical Association 39(8): 516-519.

Saye, D.J., Ogunseitan, O.A., Sayler, G.S. and Miller, R.V. 1987. Potential for transduction of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 53: 987-995.

Saye, D.J., Ogunseitan, O.A., Sayler, G.S. and Miller, R.V. 1990. Transduction of linked chromososmal genes between *Pseudomonas aeruginosa* strains during incubation *in situ* in a freshwater habitat. Applied and Environmental Microbiology 56: 140-145.

Sayre, P.S. and Miller, R.V. 1990. Bacterial mobile genetic elements: importance in assessing the environmental fate of genetically engineered sequences. Plasmid 26: 151-171.

Schleifer, K.H., Amann, R., Ludwig, W., Rothemund, C., Springer, N. and Dorn, S. 1992. Nucleic acid probes for the identification and *in situ* detection of pseudomonads. In: Galli, E., Silver, S. and Witholt, B. (eds). *Pseudomonas. Molecular Biology and Biotechnology* (pp. 105-115). American Society for Microbiology, Washington, D.C.

Schmidt, E.L. 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Science 118: 141-149.

Scholter, M., Bode, W., Hartmann, A. and Besse, F. 1992. Sensitive chemoluminescence-based immunological quantification of bacteria in soil extracts with monoclonal antibodies. Soil Biology and Biochemistry 24: 399-405.

Schroth, M.N., Hildebrand, D.C. and Starr, M.P. 1981. Phytopathogenic members of the genus *Pseudomonas* pp. 701-718 In: Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schegel, H.G. (eds). *The Prokaryotes - a Handbook on Habitats, Isolation and Identification of Bacteria*. Springer-Verlag, Berlin.

Schroth, M.N., Hildebrand, D.C. and Panopoulos, N. 1992. Phytopathogenic Pseudomonads and related plant-associated Pseudomonads. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. (eds). *The Prokaryotes* (pp. 3104-3131), Chapter 162, Vol. III (2nd Edition). Springer-Verlag Inc., New York.

Schuster, M.G. and Norris, A.H. 1994. Community-acquired *Pseudomonas aeruginosa* pneumonia in patients with HIV infection. AIDS 8(10): 1437-1441.

Scott, J., Boulton, F.E., Govan, J.R.W., Miles, R.S., McClelland, D.B.L. and Prowse, C.V. 1988. A fatal transfusion reaction associated with blood contaminated with *Pseudomonas fluorescens.* Vox Sang. 54: 201-204.

Sellwood, J.E., Ewart, J.M. and Buckler, E. 1981. Vascular blackening of chicory caused by a pectolytic isolate of *Pseudomonas fluorescens*. Plant Pathology 30: 179-180.

Sepkowitz, K.A., Brown, A.E. and Armstrong, D. 1994. Empirical therapy for febrile, neutropenic patients: Persistence of susceptibility of gram-negative bacilli to aminoglycoside antibiotics. Clinical Infectious Diseases 19(4): 810-811.

Shahata, M. A., El-Timawy, A. M. and Seddik, I. 1988. Occurrence of *Pseudomonas* infection in fowl in upper Egypt. Assiut Veterinary Medical Journal 20(39): 168-177.

Shaw, J.J. and Kado, C.I. 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the on-going disease process in a non-disruptive manner. Bio/Technology 4: 560-564.

Shaw, J.J. and Kado, C.I. 1988. Whole plant wound inoculation for consistent reproduction of black rot of crucifers. Phytopathology 78: 981-986.

Short, K.A., Doyle, J.D., King, R.J., Seidler, R.J., Stotzky, G. and Olsen, R.G. 1991. Effects of 2,4-dichlorophenol, a metabolite of a genetically engineered bacterium, and 2,4-dichlorophenoxyacetate on some microorganism-mediated ecological processes in soil. Applied and Environmental Microbiology 57: 412-418.

Silcock, D.J., Waterhouse, R.N., Glover, L.A., Prosser, J.I. and Killham, K. 1992. Detection of a single genetically modified bacterial cell in soil by using charge coupled device-enhanced microscopy. Applied and Environmental Microbiology 58(8): 2444-2448.

Simor, A.E., Ricci, J., Lau, A., Bannatyne, R.M. and Ford-Jones, L. 1985. Pseudobacteremia due to *Pseudomonas fluorescens*. Pediatric Infectious Diseases 4: 508-512.

Sisinthy, S., Rao, N.S., Saisree, L., Vipula, S., Reddy, G.N.S. and Bhargava, P.M. 1989. Isolation and identification of *Pseudomonas* spp. from Schirmacher Oasis, Antarctica. Applied and Environmental Microbiology 55: 767-770.

Smirnov, V.V., Kiprianova, E.A., Boiko, O.I., Kolesova, E. A. and Garagulya, A.D. 1991. The effect of iron ions on antifungal activity of *Pseudomonas*. Mikrobiologicheskii Zhurnal 53(3): 80- 87.

Smith, P.R. and Davey, S. 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. Journal of Fish Diseases 16: 521-524.

Som, N.C., Ghosh, B.B., Arora, G.S. and Majumdar, M.K. 1980. Isolation and identification of *Pseudomonas aeruginosa* pathogenic to insect larvae. Indian Journal of Experimental Biology 18(6): 590-593.

Sorenson, J., Skouv, J., Jorgensen, A. and Nybroe, O. 1992. Rapid identification of environmental isolates of *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida* by SDS-PAGE analysis of whole-cell protein patterns. FEMS Microbiology Ecology 101: 41-50.

Sorheim, R., Torsvik, V.L. and Goksoyr, J. 1989. Phenotypical divergences between populations of soil bacteria isolated on different media. Microbial Ecology 17: 181-192.

Spencer, R.C. 1994. Epidemiology of infection in ICUs. Intensive Care Medicine 20 (suppl 4): S2-6.

Stadt, S.J. and Saettler, A.W. 1981. Effect of host genotype on multiplication of *Pseudomonas phaseolicola*. Phytopathology 71: 1307-1310.

Stapleton, F., Dart, J.K., Seal, D.V. and Matheson, M. 1995. Epidemiology of *Pseudomonas aeruginosa* keratitis in contact lens wearers. Epidemiology and Infection 114: 395-402.

Steffan, R.J. and Atlas, R.M. 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Applied and Environmental Microbiology 55: 2185-2191.

Stenhouse, M.A. and Milner, L.V. 1992. A survey of cold-growing gram-negative organisms isolated from the skin of prospective blood donors. Transfus. Med. 2: 235-237.

Stephen, J. and Pietrowski, R.A. 1986. Bacterial Toxins. 2nd edition. American Society for Microbiology, Washington, D.C.

Stewart, G.J. 1990. The mechanism of natural transformation. In: Levy, S.B. and Miller, R.V. (eds). *Gene Transfer in the Environment (*pp. 139-164). McGraw-Hill Publishing Co, New York.

Stewart, G.J. and Carlson, C.A. 1986. The biology of natural transformation. Annual Review of Microbiology 40: 211-235.

Stone, L.S. and Zottola, E.A. 1985. Relationship between the growth phase of *Pseudomonas fragi* and its attachment to stainless steel. Journal of Food Science 50(4): 957-960.

Stoskopf, M.K. 1993. Bacterial diseases of goldfish, koi, and carp. In: Stoskopf, M.K. *Fish Medicine* (pp. 473-475), Chapter 48. W.B. Saunders Co., Philadelphia, PA.

Stotzky, G., Zeph, L.R. and Devanas, M.A. 1991. Factors affecting the transfer of genetic information among microorganisms in soil. In: Ginzburg, L.R. (ed.). *Assessing Ecological Risks in Biotechnology* (pp. 95-112). Butterworth-Heineman.

Sturman, P.J., Jones, W.L. and Characklis, W.G. 1994. Interspecies competition in colonized porous pellets. Water Research 28(4): 831-839.

Stutz, E., Kahr, G. and Defago, G. 1989. Clays involved in suppression of tobacco black rot by a strain of *Pseudomonas fluorescens*. Soil Biology and Biochemistry 21: 361-366.

Sugimoto, E.E., Hoitink, H.A.J. and Tuovinen, O.H. 1990. Oligotrophic pseudomonads in the rhizosphere: suppressiveness to *Pythium* damping-off of cucumber seedlings *(Cucumis sativus* L.). Biology and Fertility of Soils 9(3): 231-34.

Suoniemi, A., Bjorklof, K., Haahtela, K. and Romantschuk, M. 1995. Pili of *Pseudomonas syringae* pathovar syringae enhance initiation of bacterial epiphytic colonization of bean. Microbiology UK 141(Feb, Part 2): 497-503.

Surico, G. and Scala, A. 1992. Carnation - *Pseudomonas caryophylli* interaction - Population dynamics of *P. caryophylli* and other bacteria in carnation leaves and cultured cells. Petria 2: 93-106.

Suyama, K. and Fujii, H. 1993. Bacterial disease occurred on cultivated mushroom in Japan. Journal of Agricultural Science 38(2): 35-50.

Tabor, M. and Gerety, R.J. 1984. Five cases of *Pseudomonas* sepsis transmitted by blood transfusions. Lancet i: 1403.

Taylor, M., Keane, C.T. and Falkiner, F.R. 1984. *Pseudomonas putida* in transfused blood. Lancet ii:107.

Thiem, S., Krumme, M., Smith, R. and Tiedje, J. 1994. Use of molecular techniques to evaluate the survival of a microorganism injected into an aquifer. Applied and Environmental Microbiology 60: 1059-1067.

Thomas, A.W., Slater, J.H. and Weightman, A.J. 1992. The dehalogenase gene *deh1* from *Pseudomonas putida* PP3 is carried on an unusual mobile genetic element designated *DEH*. Journal of Bacteriology 174: 1932-1940.

Thomashow, L.S. and Pierson, L.S. 1991. Genetic aspects of phenazine antibiotic production by fluorescent pseudomonads that suppress take-all disease of wheat. In: Hennecke, H. and Verma, D.P.S. (eds). *Proceedings of the 5th International Symposium on the molecular genetics of plant-microbe interactions*, Interlaken, Switzerland, September, 1990 (pp. 443-449). Advances in molecular genetics of plant-microbe interactions. Vol. 1. Kluwer Academic Publishers, Netherlands.

Thomashow, L.S., Weller, D.M., Bonsall, R.F. and Pierson, L.S. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Applied and Environmental Microbiology 56(4): 908-912.

Thompson, I.P., Cook, K.A., Letheridge, G. and Burns, R.G. 1990. Survival of two ecologically distinct bacteria (*Flavobacterium* and *Arthrobacter*) in unplanted and rhizosphere soil: Laboratory studies. Soil Biology and Biochemistry 22: 1029-1037.

Thompson, I.P., Bailey, M.J., Fenlon, J.S., Fermor, T.R., Lilley, A.K., Lynch, J.M., McCormack, P.J., McQuilken, M.P., Purdy, K.J., Rainey, P.B. and Whipps, J.M. 1993a. Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugarbeet (*Beta vulgaris*). Plant and Soil 150(2): 177-191.

Thompson, I.P., Bailey, M.J., Ellis, R.J. and Purdy, K.J. 1993b. Subgrouping of bacterial populations by cellular fatty acid composition. FEMS Microbiology Ecology 102: 75-84.

Thompson, S.V., Hanson, D.R., Flint, K.M. and Vandenberg, J.D. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honey bees. Plant Disease 76: 1052-1056.

Thorn, R.G. and Tsuneda, A. 1992. Interactions between various wood-decay fungi and bacteria: antibiosis, attack, lysis, or inhibition. Reports of the Tottori Mycological Institute 30: 13-20.

Timmis, K.N. 1994. Environmental biotechnology. Current Opinion in Biotechnology 5: 247-248.

Top, E., Vanrolleghem, P., Mergeay, M. and Verstraete, W. 1992. Determination of the mechanism of retrotransfer by mechanistic modelling. Journal of Bacteriology 174: 5953-5960.

Tranel, P.J., Gealy, D.R. and Kennedy, A.C. 1993. Inhibition of downy brome (*Bromus tectorum*) root growth by a phytotoxin from *Pseudomonas fluorescens* strain D7. Weed Technology 7: 134-139.

Trevors, J.T. 1991. Development of a Microbial Profile on Selected Groups of Microorganisms to assist in evaluations under the Canadian Environmental Protection Act for the Genus: *Pseudomonas*. Environment Canada contract report KE144-0623901SS.

Trevors, J.T. and Berg, G. 1989. Conjugal RP4 transfer between pseudomonads in soil and recovery of RP4 plasmid DNA from soil. Systematic and Applied Microbiology 11: 223-227.

Trevors, J.T. and Starodub, M.E. 1987. R-Plasmid transfer in non-sterile agricultural soil. Systematic and Applied Microbiology 9: 312-315.

Trevors, J.T. and van Elsas, J.D. 1989. A review of selected methods in environmental microbial genetics. Microbiological Sciences 5: 132-136.

Trevors, J.T., van Elsas, J.D., Overbeek, L.S. and Starodub, M-E. 1990. Transport of a genetically engineered *Pseudomonas fluorescens* strain through a soil microcosm. Applied and Environmental Microbiology 56: 401-408.

Trueb, R.M., Gloor, M. and Wuthrich, B. 1994. Recurrent *Pseudomonas* folliculitis. Pediatric Dermatology 11: 35-38.

Tsai, Y-L. and Olson, B.H. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Applied and Environmental Microbiology 58: 754-757.

Tsuchiya, Y., Ohata, K.I. and Shirata, A. 1980. Pathogenicity of the causal bacteria of head rot of lettuce, *Pseudomonas cichorii*, *P. marginalis* and *P. viridiflava*, to various crop plants. Bulletin of the National Institute of Agricultural Sciences 34: 51-73.

Tsuda, M., Minegishi, K.-I. and Iino, T. 1989. Toluene transposons *Tn4651* and *Tn4653* are class II transposons. Journal of Bacteriology 171: 1386-1393.

Tsuda, M. and Iino, T. 1990. Napthalene degrading genes on plasmid NAH7 are on a defective transposon. Molecular and General Genetics 22: 33-39.

Turkovskaya, O.V., Shub, G.M. and Panchenko, L.V. 1993. Microbial purification of nonionic surfactant sewage. pp. 171-183 In: *Effluent Treatment and Waste Minimization Symposium*. Yorkshire Branch of the Institution of Chemical Engineers and the Environmental Protection Subject Group, Leeds, UK, Mar. 31-Apr. 1, 1993. Symposium Series No. 132.

Tushima, S., Hasebe, A., Komoto, Y., Carter, J.P., Miyashita, K. and Pickup, R.W. 1995. Detection of genetically engineered microorganisms in paddy soil using a simple and rapid “nested” polymerase chain reaction method. Soil Biology and Biochemistry 27: 219-227.

Tyler, S.D., Strathdee, C.A., Rozee, K.R. and Johnson, W.M. 1995. Oligonucleotide primers designed to differentiate pathogenic pseudomonads on the basis of the sequencing of 16S-23S genes coding for internal transcribed spacers. Clinical Diagnostic Laboratory Immunology 2: 448-453.

van Dyke, M.I., Gulley, S.L., Lee, H. and Trevors, J.T. 1993. Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. Journal of Industrial Microbiology 11: 163-170.

van Elsas, J.D., Dijkstra, A.F., Govaert, J.M. and van Veen, J.A. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiology Ecology 38: 151-160.

van Elsas, J.D., Govaert, J.M. and van Veen, J.A. 1988. Bacterial conjugation between pseudomonads in the rhizosphere of wheat. FEMS Microbiology Ecology 53: 299-306.

van Elsas, J.D., Trevors, J.T. and van Overbeek, L.S. 1991. Influence of soil properties on the vertical movement of genetically marked *Pseudomonas fluorescens* through large soil microcosms. Biology and Fertility of Soils 10: 249-255.

van Elsas, J.D., Trevors, J.T., Jain, D., Wolters, A.C., Heijnen, C.E. and van Overbeek, L.S. 1992. Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. Biology and Fertility of Soils 14: 14-22.

Vancura, V. 1988. Inoculation of plants with *Pseudomonas putida*. In: Vancura, V. and Kunc, F. (eds). *Interrelationships Between Microorganisms and Plants in Soil (*pp. 185-190). Elsevier Science Publishers, Amsterdam, Netherlands.

Vandenbergh, P.A. and Kunka, B.S. 1988. Metabolism of volatile chlorinated aliphatic hydrocarbons by *Pseudomonas fluorescens*. Applied and Environmental Microbiology 54: 2578-2579.

Vandenhove, H., Merckx, R., Wilmots, H. and Vlassak, K. 1991. Survival of *Pseudomonas fluorescens* inocula of different physiological stages in soil. Soil Biology and Biochemistry 23: 1133-1142.

Vasil, M.L., Pritchard, A.E. and Ostroff, R.M. 1990. Molecular biology of exotoxin A and phospholipase C of *Pseudomonas aeruginosa.* In: Silver, S., Chakrabarty, A.M., Igelewski, B. and Kaplan, S. (eds). *Pseudomonas*: *transformations, pathogenesis and evolving biotechnology.* American Society for Microbiology, Washington, D.C.

Vesaluoma, M., Kalso, S., Jokipii, L., Warhurst, D., Ponka, A. and Tervo, T. 1995. Microbiological quality in Finnish public swimming pools and whirlpools with special reference to free living amoebae: a risk factor for contact lens wearers? British Journal of Ophthalmology 79: 178-181.

Vesper, S.J. 1987. Production of pili (fimbrae) by *Pseudomonas fluorescens* and correlation with attachment to corn roots. Applied and Environmental Microbiology 53: 1397-1405.

Walter, M., Porteous, A. and Seidler, R. 1987. Measuring genetic stability in bacteria of potential use in genetic engineering. Applied and Environmental Microbiology 53: 105-109.

Wang, S.Z. 1987. Diagnosis and control of a disease in mink caused by *Pseudomonas aeruginosa*. Chinese Journal of Veterinary Medicine 13(11): 16-17.

Warburton, D.W., Bowen, B. and Konkle, A. 1994. The survival and recovery of *Pseudomonas aeruginosa* and its effect upon salmonellae in water - Methodology to test bottled water in Canada. Canadian Journal of Microbiology 40(12): 987-992.

Warren, G.J. 1987. Bacterial ice nucleation: molecular biology and applications. Biotechnology and Genetic Engineering Review 5: 107-135.

Weissenfels, W., Beyer, M. and Klein, J. 1990. Bacterial degradation of naphthalene, phenathrene, fluorene and fluoranthene by pure strains. In: *DECHEMA Biotechnology Conferences* (pp. 867-870), Vol. 3, Part B. Frankfurt, Germany. May 30-31, 1989.

Weller, D.M. and Cook, R.J. 1986. Increased growth of wheat by seed treatments with fluorescent pseudomonads, implications of *Pythium* control. Canadian Journal of Plant Pathology 8: 328-334.

Wellington, E.M.H., Cresswell, N. and Saunders, V.A. 1990. Growth and survival of streptomycete inoculants and extent of plasmid transfer in sterile and non-sterile soil. Applied and Environmental Microbiology 56: 1413-1419.

Welsh, J. and McClelland. 1990. Fingerprinting genomems using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

Wendtpotthoff, K., Backhaus, H. and Smalla, K. 1994. Monitoring the fate of genetically engineered bacteria sprayed on the phylloplane of bush beans and grass. FEMS Microbiology Ecology 15(3-4): 279-290.

Westcott, S.W. and Kluepfel, D.A. 1993. Inhibition of *Criconemella xenoplax* egg hatch by *Pseudomonas aureofaciens*. Phytopathology 83(11): 1245-1249.

White, C.P. and Dunn, H.W. 1978. Compatibility and sex specific phage plating characteristics of the TOL and NAH catabolic plasmids. Genetical Research 32: 207-213.

Wiedemann, B. and Atkinson, B.A. 1991. Susceptibility to antibiotics: Species incidence and trends. In: Lorian, V. (ed.). *Antibiotics in Laboratory Medicine*, pp. 962-1208. Williams and Wilkins, Baltimore, Maryland.

Williams, J.G.K., Kubelik, A.R., Livak, A.R., Rafalski, J.A. and Tingey, S.V. 1990. DAN polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

Williams, R.R., Dehdashti, B. and Lightner, D.V. 1992. Performance of an aquatic multispecies system in evaluating the effects of a model microbial pest control agent on nontarget organisms. Journal of Toxicology and Environmental Health 37: 247-264.

Wilson, M. and Lindow, S.E. 1993a. Effect of phenotypic plasticity on epiphytic survival and colonization by *Pseudomonas syringae*. Applied and Environmental Microbiology 59(2): 410-416.

Wilson, M. and Lindow, S.E. 1993b. Release of recombinant microorganisms. Annual Review of Microbiology 47: 913-944.

Wilson, M. and Lindow, S.E. 1994. Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. Applied and Environmental Microbiology 60(12): 4468-4477.

Wilson, M.J., Glen, D.M., Hughes, L.A., Pierce, J.D. and Rodgers, P.B. 1994. Laboratory tests of the potential of entomopathogenic nematodes for the control of field slugs (*Deroceras reticulatum*). Journal of Invertebrate Pathology 64: 182-187.

Winstanley, C., Morgan, J.A.W., Pickup, R.W., Jones, J.G. and Saunders, J.R. 1989. Differential regulation of lambda pL and pR promotors by a cI repressor in a broad-host-range thermoregulated plasmid marker system. Applied and Environmental Microbiology 55: 771-777.

Winstanley, C., Seasman, M., Pickup, R.W., Carter, I.P., Morgan, J.A.W. and Saunders, J.R. 1993. A comparison of the survival of stable and unstable chromosomally located xylE marker cassettes as an indicator of cell division within populations of *Pseudomonas putida* released into lake water and soil. Microbial Releases 2: 97-107.

Wong, P.K., Lam, K.C. and Lo, C.M. 1993. Removal and recovery of Cu(II) from industrial effluent by immobilized cells of *Pseudomonas putida* II-11. Applied Microbiology and Biotechnology 39: 127-131.

Yabuuchi, E. and Devos, P. 1995a. Minutes of the meetings of the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of the Genus *Pseudomonas* and Related Organisms. Prague, Czech Republic, 4 July 1994. International Journal of Systematic Bacteriology 45: 875.

Yabuuchi, E and Devos, P. 1995b. Minutes of the meetings of the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of the Genus *Pseudomonas* and Related Organisms. Osaka, Japan, 17 and 20 September, 1990. International Journal of Systematic Bacteriology 45: 877-878.

Yang, P., De Vos, P. Kersters, K. and Swings, J. 1993. Polyamine patterns as chemotaxonomic markers for the genus *Xanthomonas*. International Journal of Systematic Bacteriology 43: 709-714.

Youssef, H., Mourad, M.I., Karim, S.A., Hassanein, K.M. and Sadek, A. 1990. Pathogenicity of *Pseudomonas aeruginosa* to Nile fish. Assiut Veterinary Medical Journal 24(47): 108-115.

Zhou, J. and Tiedje, J. 1995. Gene transfer from a bacterium injected into an aquifer to an indigenous bacterium. Molecular Ecology 4: 613-618.

Zilli, M., Converti, A., Lodi, A., del Borghi, M. and Ferraiolo, G. 1993. Phenol removal from waste gases with a biological filter by *Pseudomonas putida*. Biotechnology and Bioengineering 41: 693-699.

Zurrer, D.A., Cook, A. and Leisinger, T. 1987. Microbial desulfonation of substituted napthalene sulfonic acids and benzene sulfonic acids. Applied and Environmental Microbiology 53: 1459-1463.

Appendix:
Considerations from the OECD "Blue Book"

The ***General Scientific Considerations***, ***Human Health Considerations*** and ***Environmental and Agricultural Considerations*** from the OECD "Blue Book" (*Recombinant DNA Safety Considerations*, OECD, 1986) are the basis of the format of the information presented in Section III of this consensus document. These considerations were also used as a reference point in the document *Analysis of Information Elements Used in the Assessment of Certain Products of Modern Biotechnology* (OECD, 1995), which identified commonalities among OECD Member countries with respect to information elements used during regulatory assessments.

The Considerations set out in the OECD "Blue Book" are as follows:

GENERAL SCIENTIFIC CONSIDERATIONS

Characteristics of Donor and Recipient Organisms

1. Taxonomy, identification, source, culture

1a Names and designations;
1b The degree of relatedness between the donor and recipient organisms and evidence indicating exchange of genetic material by natural means;
1c Characteristics of the organism which permit identification and the methods used to identify the organisms;
1d Techniques employed in the laboratory and/or environment for detecting the presence of, and for monitoring, numbers of the organism;
1e The sources of the organisms;
1f Information on the recipient organism's reproductive cycle (sexual/asexual);
1g Factors which might limit the reproduction, growth and survival of the recipient organism.

2. Genetic characteristics of donor and recipient organisms

2a History of prior genetic manipulation;
2b Characterisation of the recipient and donor genomes;
2c Stability of recipient organism in terms of relevant genetic traits.

3. Pathogenic and physiological traits of donor and recipient organisms

3a Nature of pathogenicity and virulence, infectivity, or toxigenicity;
3b Host range;
3c Other potentially significant physiological traits;
3d Stability of these traits.

Character of the Engineered Organism

4a Description of the modification;
4b Description of the nature, function and source of the inserted donor nucleic acid, including regulatory or other elements affecting the function of the DNA and of the vector;
4c Description of the method(s) by which the vector with insert(s) has been constructed;
4d Description of methods for introducing the vector-insert into the recipient organism and the procedure for selection of the modified organism;
4e Description of the structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism;
4f Characterisation of the site of modification of the recipient genome. Stability of the inserted DNA;
4g Frequency of mobilisation of inserted vector and/or genetic transfer capability;
4h Rate and level of expression of the introduced genetic material. Method and sensitivity of measurement;
4i Influence of the recipient organism on the activity of the foreign protein.

HUMAN HEALTH CONSIDERATIONS

Characteristics of the Engineered Organism

1. Comparison of the engineered organism to the recipient organism regarding pathogenicity;
2. Capacity for colonisation;
3. If the organism is pathogenic to humans (or to animals if appropriate):

    3a Diseases caused and mechanism of pathogenicity including invasiveness and virulence;
    3b Communicability;
    3c Infective dose;
    3d Host range, possibility of alteration;
    3e Possibility of survival outside of human host;
    3f Presence of vectors or means of dissemination;
    3g Biological stability;
    3h Antibiotic-resistance patterns;
    3i Toxigenicity;
    3j Allergenicity.

Health Considerations Generally Associated with the Presence of Non-viable Organisms or with the Products of rDNA Processes

4. Toxic or allergenic effects of non-viable organisms and/or their metabolic products;
5. Product hazards.

Management of Personnel Exposure

6. Biological Measures:

   6a Availability of appropriate prophylaxis and therapies;
   6b Availability of medical surveillance.

7. Physical and organisational measures.

ENVIRONMENTAL AND AGRICULTURAL CONSIDERATIONS

Ecological Traits relating to the Donor and Recipient

1a Natural habitat and geographic distribution. Climatic characteristics of original habitats;
1b Significant involvement in environmental processes;
1c Pathogenicity - host range, infectivity, toxigenicity, virulence, vectors;
1d Interactions with and effects on other organisms in the environment;
1e Ability to form survival structure (e.g., seeds, spores, sclerotia);
1f Frequency of genotypic and phenotypic change;
1g The role of the genetic material to be donated in the ecology of the donor organism;
1h The predicted effect of the donated genetic material on the recipient organism.

Application of the Engineered Organism in the Environment

2a Geographical location of site, physical and biological proximity to man and/or any other significant biota;
2b Description of site including size and preparation, climate, temperature, relative humidity, etc.;
2c Containment and decontamination;
2d Introduction protocols including quantity and frequency of application;
2e Methods of site disturbance or cultivation;
2f Methods for monitoring applications;
2g Contingency plans;
2h Treatment procedure of site at the completion of application.

Survival, Multiplication and Dissemination of the Engineered Organism in the Environment

Detection, identification and monitoring techniques

3a Description of detection, identification and monitoring techniques;
3b Specificity, sensitivity and reliability of detection techniques;
3c Techniques for detecting transfer of the donated DNA to other organisms.

Characteristics affecting survival, multiplication and dissemination

4a Biological features which affect survival, multiplication or dissemination;
4b Behaviour in simulated natural environments such as microcosms, growth rooms, greenhouses, insectaries, etc.;
4c Known and predicted environmental conditions which may affect survival, multiplication, dissemination.

Interactions of Engineered Organism(s) with Biological Systems

Target and non-target populations

5a Known and predicted habitats of the engineered organism;
5b Description of the target ecosystems and of ecosystems to which the organism could be disseminated;
5c Identification and description of target organisms;
5d Anticipated mechanism and result of interaction between the engineered organism and the target organism(s);
5e Identification and description of non-target organism(s) which might be exposed.

Stability

6a Stability of the organism in terms of genetic traits;
6b Genetic transfer capability;
6c Likelihood of post-release selection leading to the expression of unexpected and undesirable traits by the engineered organism;
6d Measures employed to ensure genetic stability, if any;
6e Description of genetic traits which may prevent or minimise dispersal of genetic material.

Routes of dissemination

7a Routes of dissemination, physical or biological;
7b Known or potential modes of interaction, including inhalation, ingestion, surface contact, burrowing and injection.

Potential Environmental Impacts

Potential effects on target and non-target organisms

8a Pathogenicity, infectivity, toxigenicity, virulence, vector of pathogen, allergenicity, colonisation;
8b Known or predicted effects on other organisms in the environment;
8c Likelihood of post-release shifts in biological interactions or in host range.

Ecosystems effects

9a Known or predicted involvement in biogeochemical processes;
9b Potential for excessive population increase.

QUESTIONNAIRE TO RETURN TO THE OECD

The **Consensus Document on Information Used in the Assessment of Environmental Applications Involving *Pseudomonas*** is one in a series of OECD "consensus documents" containing information for use during a regulatory assessment of a particular microorganism, or of a new plant variety developed through modern biotechnology. These documents have been developed with the intention that they will be updated regularly to reflect scientific and technical developments.

Users of this document are invited to provide the Environmental Health and Safety Division with relevant new scientific and technical information, and to make proposals for additional areas related to this subject which ought to be considered in the future. This questionnaire is pre-addressed (see reverse). Respondents may either mail this page (or a photocopy) to the OECD, or forward the information requested via fax or E-mail.

OECD Environment Directorate
Environmental Health and Safety Division
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*For more information about the Environmental Health and Safety Division and its publications (many of which are available electronically at no charge), consult the OECD's World Wide Web site**: http://www.oecd.org/ehs/***

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1. Did you find information in this document useful to your work?
   ☐ YES ☐ NO

2. Please specify the type of work you do:
   ☐ REGULATORY ☐ ACADEMIC ☐ INDUSTRY ☐ OTHER (please specify)

3. Please suggest changes or additions that should be considered when this document is updated.

4. Are there other areas related to this subject which should be considered when the document is updated?

Name:.....
Institution or company:.....
Address:.....
City: .....Postal code:..... Country:.....
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OECD Environment Directorate
Environmental Health and Safety Division
2, rue André Pascal
75775 Paris Cedex 16
France

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APPENDIX 3

Compilation of U.S. EPA *Pseudomonas fluorescens* registration review decisions.

Pseudomonas fluorescens Final Work Plan
Registration Review - Case 6006
January 2008

Approved by: _____

Janet Andersen, Director
Biopesticides and Pollution Prevention Division

Date: JAN 7 2008

Introduction:

This is EPA's ***Final Work Plan*** for the registration review of *Pseudomonas fluorescens.* The work plan includes the expected registration review time line. The work plan also addresses public comments received concerning the ***Preliminary Work Plan*** in the ***Summary Document*** which was posted in the *Pseudomonas fluorescens* review docket, or any other comments concerning initial docket postings. The ***Summary Document*** provided information on what EPA knows about the pesticide and what additional risk analyses and data or information the Agency believes are needed to make a registration review decision.

The Agency is implementing the new Registration Review program and will review each registered pesticide every 15 years to determine whether it continues to meet the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) standard for registration. Changes in science, public policy, and pesticide use practices will occur over time. The registration review program is intended to make sure that, as the ability to assess risk evolves and as policies and practices change, all registered pesticides continue to meet that statutory standard. The public phase of registration review begins when the initial docket is opened for each case. Information on this program is provided at: http://www.epa.gov/oppsrrd1/registration_review/.

Summary of Comments Received on Preliminary Work Plan:

The *Pseudomonas fluorescens* registration review docket was open for a 90-day comment period beginning September 19, 2007. Two comments were received which are summarized below. These comments did not change the work plan or timeline set out in the preliminary work plan. Further, this document makes final the work plan for the *Pseudomonas fluorescens* registration review process.

Comment EPA-HQ-OPP-2007- 0014 **:** Ravi Vohra submitted a comment pertaining to four registration review dockets (EPA-HQ-OPP-2007-0569; EPA-HQ-OPP-2007-0509; EPA-HQ-OPP-2007-0567, EPA-HQ-OPP-2007-0566), requesting farmers and pesticide makers work together to label fruits that have been sprayed with a certain type of pesticides to let the consumers know of side effects, if any. She states that she "has personally researched each pesticide and most of them have some minor side effects that would be beneficial to include on a fruit or vegetable that has been sprayed with pesticide."

Response: It is noted that all of the active ingredients (Farnesol, Nerolidol, Chitin, Chitosan, *Pseudomonas syringae,and Pseudomonas fluorescens)* that were subject to this comment have been exempted from the requirements of a tolerance. When these pesticides are used in accordance with the product label, adverse effects to humans, wildlife, and the environment are not anticipated. The labeling of fruits and vegetables is not within the EPA's jurisdiction, but is within FDA's jurisdiction.

Comment EPA-HQ-OPP-2007-0015: Reza Islam submitted a comment pertaining to three registration review dockets (EPA-HQ-OPP-2007-0509; EPA-HQ-OPP-2007-0567, EPA-HQ-OPP-2007-0566), expressing her opinion that the pesticides in question meet the standards of the Federal Insecticide, Fungicide, and Rodenticide Act., based on prior determinations concerning these pesticides by EPA and that these pesticides are found naturally in the common foods we eat and in plants. She expresses support for the use of these specific pesticides and indicates that they are a useful tool in ensuring the health and quality of plants and crops.

Response:
No response necessary.

Risk Assessment and Data Needs:

These *Pseudomonas fluorescens* strain A506 and strain 1629RS occur naturally in the environment. The specific registered strains demonstrate a low toxicity profile. Based on these factors and the data submitted, no adverse ecological risks are expected as a result of exposure to these microorganisms, when used as labeled. It is anticipated that we will need further product identity data concerning the cell collection identity numbers for *Pseudomonas fluorescens* strain A506 and strain 1629RS. These product identity data are being requested at this time. If additional information is submitted that warrants further risk assessments, the Agency will then conduct any necessary risk assessment(s).

Human Health Risk:

Pseudomonas fluorescens strain A506 and strain 1629RS

Based on previously completed reviews dated March 5, 1992 of human health assessment data and summary risk assessments dated September 1, 1992, the Agency anticipates that no additional human health effects data are required for the currently registered sites for *Pseudomonas fluorescens strain* A506 (ATCC) and strain 1629RS. Reviews of toxicity/pathogenicity studies, of public literature, and of scientifically based data waiver requests, place this pesticide in Toxicity Category III and IV, which pose minimal risk to health and the environment when the pesticides are used as labeled. For additional information, refer to the Fact Sheet and Supplementary Information for *Pseudomonas fluorescens* strain A506 and strain 1629RS in Parts II and IV in this document.

The biological pesticides, *Pseudomonas fluorescens* strain A506, *Pseudomonas fluorescens* strain 1629RS and *Pseudomonas syringae* 742RS are exempted from the requirement of a tolerance in or on all raw agricultural commodities when applied as a frost protection agent or biological control agent to growing agricultural crops in accordance with good agricultural practices (40 CFR 180.1114 57 FR 42700, Sept. 16, 1992). This tolerance was reassessed on June 3, 2002 and it was determined that it meets the FQPA 1996 safety standard.

Ecological Effects:

Based on use patterns, low exposure levels, and low toxicity potential, no additional ecological effects or environmental data are required for the *Pseudomonas fluorescens* currently in labeled pesticide products discussed in this document.

The registered *Pseudomonas fluorescens* strains are used as frost protection or biological control agents. There is no comparable activity on vertebrate or invertebrate species. In addition, there is no evidence of hazard to listed threatened or endangered species and/or effects on critical habitat based on data obtained from guideline studies on non-target species and/or a review of the available literature. As a result, the Agency has determined that the registered uses of *Pseudomonas fluorescens* strain A506 and 1629RS will have "No effect" (NE) on endangered or threatened terrestrial or aquatic species, nor designated critical habitat as listed by the U.S. Fish and Wildlife Service (USFWS) and the National Oceanic and Atmospheric Administration (NOAA).

Timeline:

EPA has created the following estimated timeline for the completion of the *Pseudomonas fluorescens* registration review.

Activities	**Estimated Month/Year**
Phase 1: Opening the docket	
Open Public Comment Period for *Pseudomonas fluorescens* Docket	September 2007
Close Public Comment Period	November 2007
Phase 2: Case Development	
Develop Final Work Plan (FWP)	January 2008
Phase 3: Registration Review Decision	
Open Public Comment Period for Proposed Reg. Review Decision	April 2008
Close Public Comment Period	June 2008
Final Decision and Begin Post-Decision Follow-up	August 2008
Total (years)	1

Next Steps:

The proposed Registration Review Decision for *Pseudomonas fluorescens* will be developed and placed in Docket EPA-HQ-2006-0567 for public comment.

APPENDIX 4

U.S. Army Corps of Engineers zebra mussel chemical control guide.

ERDC/EL TR-00-1

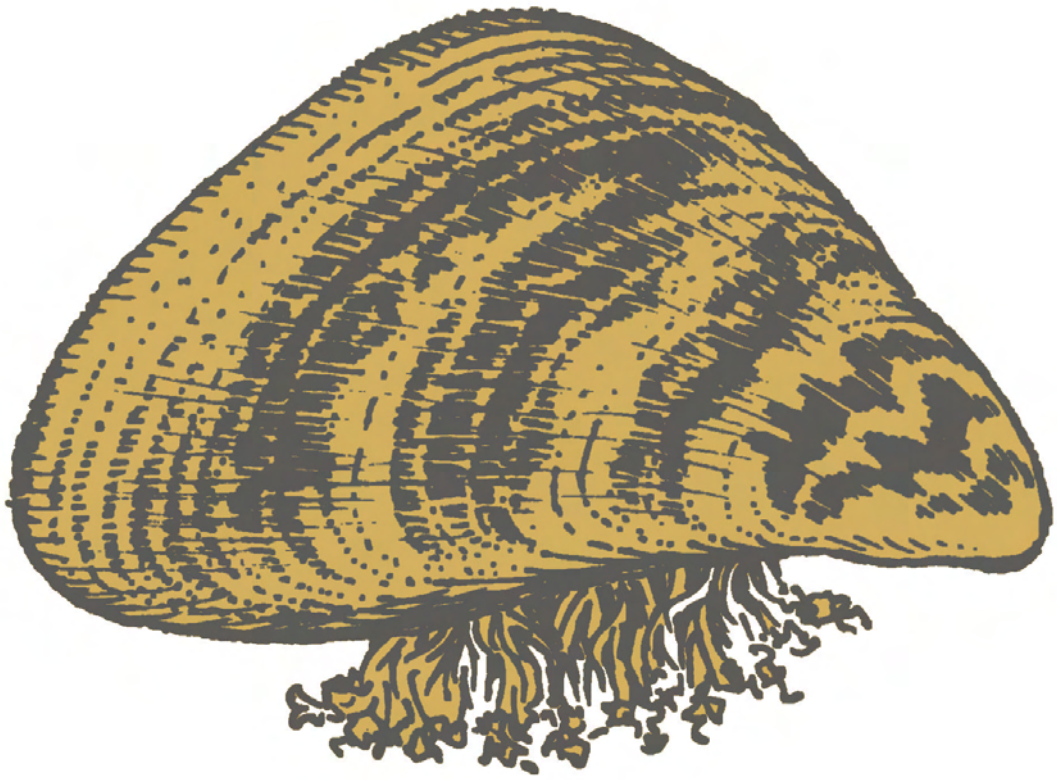
US Army Corps of Engineers®
Engineer Research and Development Center

Zebra Mussel Research Program

Zebra Mussel Chemical Control Guide

Susan L. Sprecher and Kurt D. Getsinger

January 2000



Environmental Laboratory

Zebra Mussel
Research Program

ERDC/EL TR-00-1
January 2000

Zebra Mussel Chemical Control Guide

by Susan L. Sprecher, Kurt D. Getsinger

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Final report

Approved for public release; distribution is unlimited

Prepared for U.S. Army Corps of Engineers
Washington, DC 20314-1000

Under Work Unit 33156

Army Engineer Research and Development Center Cataloging-in-Publication Data

Sprecher, Susan L.
Zebra mussel chemical control guide / by Susan L. Sprecher, Kurt D. Getsinger ; prepared for U.S. Army Corps of Engineers.
114 p. : ill. ; 28 cm. -- (ERDC/EL ; TR-00-1)
Includes bibliographic references.
1. Zebra mussel -- Control. 2. Dreissena -- Control. 3. Dreissenidae — Control. 4. Molluscicides. I. Getsinger, Kurt D. II. United States. Army. Corps of Engineers. III. U.S. Army Engineer Research and Development Center. IV. Environmental Laboratory (U.S.) V. Zebra Mussel Research Program (U.S.) VI. Title. VII. Series: ERDC/EL TR ; 00-1.
TA7 E8 no.ERDC/EL TR-00-1

Contents

Preface

The work reported herein was conducted as part of the Zebra Mussel Research Program (ZMRP) under Work Unit 33156, "Aquatic Molluscicide Use Guide." The ZMRP is sponsored by Headquarters, U.S. Army Corps of Engineers (HQUSACE), and is assigned to the U.S. Army Engineer Research and Development Center (ERDC) under the purview of the Environmental Laboratory (EL), Vicksburg, MS. The HQUSACE Technical Monitors for ZMRP are Joe Wilson, Pete Juhle, and Cheryl Smith.

The purpose of this report is to provide guidance to Corps District and Project personnel on the selection and use of registered chemicals available for control of zebra mussels. To assist in the selection process, the various types of compounds that were registered as molluscicides at the time that this report was written are summarized with information on use strategies and application rates.

The Principal Investigator for this study was Dr. Kurt D. Getsinger, Ecosystem Processes and Effects Branch (EPEB), Environmental Processes and Effects Division (EPED), EL, under the general supervision of Dr. Robert H. Kennedy, Acting Chief, EPEB; Dr. Richard E. Price, Chief, EPED; and Dr. John W. Keeley, Acting Director, EL. Dr. Edwin A. Theriot, EL, was Program Manager of ZMRP. This report was written by Drs. Susan L. Sprecher and Getsinger, EPED. Technical reviews of this report were provided by Dr. H. E. Tatem and Mr. R. M. Stewart, EPED.

At the time of publication of this report, Dr. Lewis E. Link was Acting Director of ERDC, and COL Robin R. Cababa, EN, was Commander.

This report should be cited as follows:

Sprecher, S. L., and Getsinger, K. D. (2000). "Zebra mussel chemical control guide," ERDC/EL TR-00-1, U.S. Army Engineer Research and Development Center, Vicksburg, MS.

1 Introduction

Background

Control and prevention of macrofouling caused by the freshwater zebra mussel, *Dreissena polymorpha* (Pallas), have been major concerns of managers of all types of water delivery systems since shortly after the arrival of this nuisance species in North America in 1985 (Hebert, Muncaster, and Mackie 1989). The types of operating systems and system components that can be expected to undergo zebra mussel infestations and fouling include those associated with the management and control of inland waterways; potable water treatment; agriculture; industry; and power generation (McMahon, Ussery, and Clarke 1994; Claudi and Mackie 1994).

The threat to lock structures, navigation dams, reservoir control structures, vessel locks, stream level gauging systems, pumping stations, drainage structures, and other structures present in navigable waterways is of particular interest to the U.S. Army Corps of Engineers. The Corps also owns and operates 75 hydroelectric power plants in the United States, all of whose components (intake structures, cooling water, transducers, gauging stations, project irrigation, fire prevention lines, etc.) have been identified as being susceptible to zebra mussel fouling (McMahon and Tsou 1990; Neilson 1992)[1]. Generally, facilities that can be expected to be affected include hydropower dams, nonpower dams, navigation locks, fossil-fueled and nuclear-fueled generating plants, certain types of recreation sites (boat ramps, beaches), and miscellaneous other sites (Tippit, Cathey, and Swor 1993).

In North America, as in Europe, chemical applications to water have been the most commonly used method of zebra mussel treatment and control for internal and closed systems (Claudi and Mackie 1994). Numerous organic and inorganic chemicals are toxic to zebra mussels and can provide versatile, easy to implement, and cost-effective ways to deal with established infestations and to prevent new ones from occurring. Chemicals are expected to be a major control method and to be used as part of integrated control programs in the future. While these treatments can be designed to protect whole systems, their major drawback is the requirement for safe discharge in compliance with environmental regulations.

[1] In 1997 it was estimated that commercial power plants in the Great Lakes region spend $350,000 per year to clear away zebra mussel infestations from these types of areas (Jeffrey Reuter, US CoChair of the Council of Great Lakes Research Managers (CGLRM), address to CGLRM, 1 and 2 November 1997).

Since the arrival of the zebra mussel, a number of chemicals with previously known or newly discovered molluscicidal properties have been proposed for deployment against this highly invasive organism. This report describes basic guidelines for the use of those compounds that are currently registered with the United States Environmental Protection Agency (USEPA) for zebra mussel control, and includes a summary of the important registration process.

Chemical Control of the Zebra Mussel

Mussel life cycle and behavior influence the strategies and tactics of chemical control, as well as choice of molluscicidal compound. Zebra mussels cannot survive in saline conditions but are well adapted to water temperatures (12 ºC to 32 ºC (55 ºF to 90 ºF)), pH range ($6.5 to >8), and turbidity levels that can be found in the Great Lakes and many U.S. riverine environments (Claudi and Mackie 1994; Figure 1). Spawning occurs in spring when water temperatures rise above 12 ºC and can continue into October. Females release up to 30,000 planktonic (free-swimming) larvae, called veligers, which move with water currents and grow up to 1.3 cm (0.5 in.) in the first half year. These settle in colonies and attach to firm surfaces by means of secreted strands called byssal threads. Densities can reach 500,000 per square meter (46,500 or more per square foot), and individual life spans are 3 to 5 years. Zebra mussels are filter feeders, opening their shells to allow ingestion of particulates. When their sensitive chemoreceptors alert them to certain toxins in the environment, they have the ability to maintain shell closure for up to 2 weeks and thereby remain immune to certain biocide contact. Not all molluscicides evoke this response, however.

The application of chemical molluscicides in the field is limited by several considerations. Firstly, a method must be judged by how well it removes or kills the various life stages of the zebra mussel. Secondly, any chemical control method used must not be harmful to natural fisheries and aquatic ecosystems and must also be eventually compatible with possible potable water use. Thus, flow-through systems may require a different suite of chemicals than is possible in static or closed systems where there is no release to the environment. Since chemical control is most suitable for application to problems in closed systems and internal piping, it is much less effective in treatment of external surfaces where it may be impossible to maintain required treatment concentrations and contact times of the compound. Thus, current chemical options are not available for treating and reducing densities of zebra mussels in source waters, such as lakes, rivers, and streams. In these areas nonchemical methods are more suitable. Finally, use of the material must be cost-effective.

Chemicals identified for zebra mussel control have been derived mainly from water treatment compounds and antifouling biocides and biodispersants. Chlorine has been used for nearly a hundred years in drinking water disinfection, where its properties and behavior in effluent are well known, and it has been the primary chemical for zebra mussel control in Europe. In contrast, molluscicidal properties have been associated only recently with endothall, a compound used for several decades as an aquatic herbicide. Investigation of toxicity to both the target and nontarget organisms in the aquatic environment is the first step in the ongoing

Figure 1. Zebra mussel distribution (from *Dreissena!*, Vol 9(3), Summer 1998, 8-9, courtesy of New York Sea Grant) (for a current version of the map please see: *http://www.cce.cornell.edu/seagrant/nansc/zmaps.htg*)

effort to identify more compounds that will be effective against zebra mussel. While oxidizers, and particularly the various forms of chlorine, continue to be the most commonly used of the chemical controls, additional compounds have been registered; and more continue to be tested in the search for environmentally sound and effective treatment of this pest.

Chemical applications can be used for both proactive treatment, to ward off settlement of zebra mussels and subsequent fouling before they occur, and for re-active treatment, where clean-up measures are used to remove zebra mussels already at nuisance levels and disrupting system function. It has been suggested that reactive systems or procedures are adequate if 1 year's worth of shell buildup and

fouling can be tolerated by the system, allowing for the minimum of an annual purging (Claudi and Mackie 1994). Both oxidizing and nonoxidizing chemicals are suitable for this type of application. Where macrofouling buildup or the "legacy" problems of disposal of dead mussels and shells (Allen 1994) cannot be tolerated, however, proactive treatments of nonoxidizing chemical are more commonly used. These create environments hostile to the settlement stage of the zebra mussel larvae (the veliger) and maintain inviable conditions that prevent adult zebra mussel translocation and settlement. Both approaches can be combined into a single strategy.

The goal of any chemical control program is to choose chemicals that will be effective, work rapidly, and have a minimal environmental impact. Treatment chemicals can be categorized as oxidizing (electron acceptor) and nonoxidizing compounds, with different properties and requirements. Since these groupings also generally differentiate between nonproprietary versus proprietary and organic versus inorganic compounds, they are followed in this guide to describe the chemistry of molluscicide compounds and give directions for the use of each compound. Further guidance for designing a control program using chemical molluscicides for a facility or installation is given by Claudi and Mackie (1994). They provide a detailed description of chlorination strategies and outline criteria for effective chemical application in general.

Oxidizing Molluscicides

Several compounds with toxic biocidal oxidizing activity that are already widely used as disinfectants in treatment of drinking water and wastewater and in power plant facilities to remove slime and biofilms are highly effective on zebra mussels. The environmental effects and requirements for safe discharge are well understood by users and regulators. While oxidizers present problems because of their corrosive effects on metals, their low cost makes them very attractive in mussel control programs.

The major types of oxidants frequently used for chemical control of biofouling and available as generic chemicals for molluscicide use are listed in Van Benschoten et al. (1993):

a. Chlorine (gas, liquid sodium hypochlorite, powdered calcium hypochlorite).

b. Chlorine dioxide (ClO_2).

c. Chloramines, such as monochloramine (NH_2Cl).

d. Ozone (O_3).

e. Hydrogen peroxide (H_2O_2).

f. Bromine.

g. Permanganates (MnO_4^-), such as potassium permanganate.

In general they have similar modes of action based on the oxidation of organic matter, which leads to toxic and lethal effects. They are suitable for use in preventative treatment, where they are added to a system throughout the breeding season at from 0.1 to 0.5 mg L^{-1} (ppm) total residual oxidant (TRO) to prevent settling. For reactive treatments, continuous application of 0.5 to 1.0 mg L^{-1} TRO for 2 to 4 weeks can eliminate established adult colonies, but concentration and contact time required depends on temperature, water chemistry, and physiological state of the zebra mussels. Mussels do detect oxidants, and shell closure for up to 2 weeks may reduce efficacy in adults (Claudi and Mackie 1994).

While chlorine dominates all chemical use for zebra mussels, there has been concern that there will be additional restrictions on its discharge in the future due to its nonselectivity and its formation of undesirable by-products such as trihalomethanes (THMs) and chloramines upon coming into contact with organic compounds in open water. This will change the picture for chemical control of zebra mussel. Dechlorination can be achieved by addition of sodium sulfite (Barton 1993). While ozone and hydrogen peroxide are not dealt with in this user guide, toxicity to zebra mussels is summarized by Electric Power Research Institute (EPRI) (1993).

Nonoxidizing Molluscicides

Most of these chemicals were originally developed for bacterial disinfection and algae control in water treatment systems (Claudi and Mackie 1994). They include organic film-forming antifouling compounds, gill membrane toxins, and nonorganics. The proprietary formulations have a higher per-volume cost than oxidizing chemicals but remain cost-effective due to lower use rates and rapid toxicity. They often can provide better control of adult mussels due to the inability of mussels to detect them; because shells remain open, shorter exposures are required. Most are easy to apply and do not present corrosion problems for metal components. Although most compounds are biodegradable, detoxification or deactivation may be required to meet State and Federal discharge requirements; but there is virtually no formation of toxic by-products (McMahon, Shipman, and Long 1993).

Intermittent, periodic, or semicontinuous applications rather than continuous applications of nonoxidizing compounds for adult mussel control adds to their cost-effectiveness (Netherland 1997). Usually treatment is on a periodic basis for 24 hr or less during the warm-water season to remove newly settled mussels or adults, with two to three applications per year: early in the season, at peak veliger activity, and when evidence of settlement is first seen. If they are used in coordination with monitoring programs that provide accurate veliger and mussel settlement data, frequency of application can be minimized (Green 1995). Water temperature helps determine treatment concentration and length of exposure required (Claudi and Mackie 1994; Green 1995).

Within the nonoxidizing molluscicides there are several groups of compounds:

a. *Quaternary ammonium compounds, polyquaternary ammonium compounds, or polyquats*. Quaternary ammonium compounds (QACs) are organic salts that have a wide variety of uses in industry. They have been used as coagulants and flocculants in potable water since the late 1960's, and have American National Standards Institute/National Sanitation Foundation (ANSI/NSF) Standard 60 (1997) certification for this use. Several of these have been used for control of Asian clam (*Corbicula fluminea*). They are also effective in controlling mollusk fouling in once-through industrial cooling systems, and recently received Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)/USEPA registration for use as molluscicides in municipal water (Blanck, Mead, and Adams 1996). QACs are cationic surfactants, and it is their surface-binding activity that produces antifouling biocidal effects. Two major types of chloride-ion-containing QACs are used commercially in the molluscicides CLAM-TROL CT-1™ and MACROTROL™ 9210 and Calgon H-130M, and BULAB® 6002 contains a poly-QAC (polyquat) (EPRI 1993). QACs also adsorb strongly to sediments, clay particles, organic matter, and other negatively charged surfaces, so that water column concentrations are normally very low (EPRI 1993). Where QACs are released directly into a receiving system, they may require detoxification by adsorption onto bentonite clay or other agents, but are not harmful to aquatic organisms once they are bound to anionic substances (Dobbs et al. 1995).

b. *Aromatic hydrocarbons*. Compounds such as BULAB® 6009 and MEXEL 432™ also have molluscicide properties due to their surfactant and anti-macrofouling activity.

c. *Endothall*. This herbicidal compound, long used against aquatic nuisance plants, has been formulated as EVAC® and registered to control zebra mussel.

d. *Metals and their salts*.

   (1) Copper ions have long been known to be toxic to planktonic or microbial organisms in water, and yet not pose a threat to humans due to binding and inactivation in sediments. These properties are put to use in the deployment of copper ions via the MACROTECH system for zebra mussel control. Various copper and zinc ions are major components of antifouling coatings and chemicals (Race and Kelly 1997).

   (2) Potassium, potash, and potassium chloride have flocculant activity that is able to precipitate various life stages of the zebra mussel out of water (Fisher et al. 1991).

The Use Guide Outline

In the following chapters of this guide, each molluscicide compound is described using the topics in the following format. Here, the topics are introduced with general information and definitions of terms related to how the compounds are used. Refer to these definitions when assessing the guidelines for individual compounds. Many of these concepts are further defined and discussed by Claudi and Mackie (1994).

The source of information on individual compounds has been primarily the product label and its Materials Safety and Data Sheet (MSDS). Both of these documents are available from the manufacturer or vendor (and may be posted on the Internet), and are required by law to be in the possession of the user at the time of molluscicide use.

Chemical Name and Formulations

This topic gives the compound name, brand name of proprietary or commercial formulation, and manufacturer or supplier. Where compound is generic, e.g., chlorine, no supplier is given.

Mode of Action

This topic describes the way in which the compound acts against mussels to produce a toxic or inhibitory effect.

Application Strategies

Typical systems for applying chemicals to systems and sites usually require specific chemical feed equipment, piping for chemical transport, diffusors to introduce chemical to the water, and areas for chemical storage or generation (Lawrence 1997). A variety of treatment timings can be used. Claudi and Evans (1993) summarize them as *reactive* strategies, used after zebra mussels have become established in a raw water system or have fouled external structures, and *proactive* strategies designed to prevent settlement:

a. *End-of-season*. Targets adult mussels. Chemical is applied for a period sufficient to kill all adults established in the system at the end of the breeding season; thus, the system must be able to tolerate one season of fouling. Oxidizing or nonoxidizing chemicals may be used. Oxidizing chemicals are expected to require dosing at high levels for at least 2 weeks to overcome mussel closure. Ten to twenty percent of individuals are expected to survive, and prolonging treatment to achieve 100 percent mortality may be impractical.

b. *Periodic*. Targets adult mussels on a regular basis; usually carried out when densities and size of adults remain low, so that debris removal is lessened. System must be able to accept some macrofouling. It is not

necessary to achieve complete (100 percent) mortality. Oxidizing or non-oxidizing chemicals can be used.

c. *Ongoing intermittent/(continuous pulse)*. Low levels of chemicals target postveligers to prevent infestation. Since postveligers are more susceptible than adult mussels, lower concentrations can be used; however, these will then not control established adults. For use in clean systems where no plugging can be tolerated (e.g., thin piping). Little debris produced.

d. *Continuous*. To discourage all postveliger settling. For use where there is no tolerance of obstruction or fouling; lower concentrations can be used but they must be constant. Established adults affected only if chemical applied all season. Carried out only with oxidizing chemicals.

Treatment strategies also involve the type of application and the extent of the system treated at any one time. There are several options, depending on the system configuration and location of current or potential problems:

a. *Entire raw water treatment*: Addition of chemical to the forebay or injected into suction or discharge of system pump piping.

b. *Entire system treatments*: Addition of chemical so that it is present and circulating in all water within the operating system.

c. *Forebay treatments*: Treatment of water in a holding area before it is brought into the main operating system.

d. *Targeted treatments*: Addition or application of chemical to only certain portions of the complete operating system or parts of multiple systems.

e. *Recirculation treatments*: Treat forebay, then isolate forebay from incoming water and recirculate through system.

Where applicable, closed-loop systems may be set up to reduce the amount of chemical needed per application.

Timing of Application

The efficiency of many strategies is enhanced if their timing is coordinated with veliger and mussel settlement data collection.

Generally, chemical controls to prevent settling and infestation need to be only over spawning periods, defined as water temperatures greater than 55 to 61 ºF (12 to 16 ºC). Continual application of molluscicides is recommended at this time for maximum effectiveness in intake structures (McMahon and Tsou 1990).

a. *Off-line*: While a system or portion of a system is not in operational mode.

b. *On-line*: While a system is functioning.

Application Rates

A wide range of concentration/exposure time combinations can be effective. Many are reported in the literature and in product information, but much is still being found out on a trial and error basis.

Maximum Water Concentration

The Clean Water Act requires that registered biocides discharged to waters of the United States from a point source must be regulated such that water quality-based effluent limits (WQBELs) for that biocide are established in a National Pollutant Discharge Elimination System (NPDES) permit to meet State water quality standards. There must also be compliance with each State's Antidegradation Policy. Thus, discharge limits of the chemical control compound depend on local, State, and Federal water restrictions as permitted under the NPDES program to regulate the amount of pollutants that may be discharged to waters by each discharger. Effluent guidelines are technology-based and are usually given on a case-by-case basis for individual facilities.

While commercial molluscicide labels may include specific NPDES discharge limits for that compound, many labels point out that the user must obtain an NPDES permit from the appropriate State/Tribal agency or USEPA Regional Office and comply with State water quality requirements.

Products registered as pesticides by the USEPA must be handled and applied within the limits of the label instructions.

Although most molluscicides are biodegradable, some detoxification or deactivation may be required to meet State and Federal discharge requirements. See the topic "Adjuvant/Detoxicant/Deactivant Use."

Use Restrictions

Discharge restrictions and limitations on downstream use of treated water are discussed under this topic, along with permit requirements.

Timing of Results

This topic describes how rapidly zebra mussels are affected. Monitoring may be required (see "Field Instructions and Guidance on Operational Applications").

Toxicological Data

Signal Word: The USEPA-assigned signal word indicates approximately how toxic a pesticide product is. Products that are highly toxic must display on the label the signal words DANGER-POISON along with a skull and crossbones symbol. Products that display only the signal word DANGER are corrosive and can cause irreversible eye damage or severe skin injury. Products that display the signal word WARNING are moderately toxic or can cause moderate eye or skin

irritation. Products that display the signal word CAUTION are slightly toxic or may cause slight eye or skin irritation.

Aquatic toxicology: Data for the effects of the compound on various freshwater organisms. Aquatic toxicity levels are usually reported as LC_{50}, which is the concentration lethal to 50 percent of test organisms.

Precautions

This topic gives pertinent information on precautions to take when handling the compound in its undiluted and dilute states. Also refer to MSDS for personal protective equipment information.

Field Instructions and Guidance on Operational Applications

It is recommended that before a full-scale application of a treatment or treatment system, an onsite performance test be run incorporating site conditions of water temperature and chemistry and other local conditions. These factors will affect molluscicide performance (Allen 1994).

Optimal timing and efficacy of chemical control treatments can be determined using biobox monitors set up in various configurations to test presence or settlement of veligers. These are chambers connected to a side stream of the water system that allow sampling of incoming veligers or determination of toxicity to adults preseeded into the boxes. Molluscicides can be injected into the water supply at a point midway between two side-stream monitors at preestablished time intervals to evaluate treatment efficacy (Claudi and Mackie 1994; Green 1995). Data collected before, during, and after application will show effect of treatment.

Adjuvant/Detoxicant/Deactivant Use

Although most molluscicides are biodegradable, some detoxification or deactivation may be required to meet State and Federal discharge requirements. Deactivation compounds may be recommended or be required by the label during molluscicide use or before discharge.

Proprietary deactivants or detoxicants are available. Bentonite clay in a dry or slurry form is a standard agent for several of the nonoxidizing compounds, added to the system discharge upstream of its outlet to the environment. Binding properties of the clay generally render the biocide inactive.

Application Techniques

This topic gives special instructions for adding the compound to the system and maintaining it.

Antidote Information

This topic gives brief emergency instructions, including phone numbers for companies that can supply treatment information.

References

Technical references for additional information are provided for each molluscicide.

Additional Chemicals

Numerous pesticide compounds in addition to the ones presented in this guide have been suggested or investigated for zebra mussel control and are discussed in a variety of research and product information literature. However, most are currently not in common use, either because they are less effective on zebra mussels, harmful to native bivalves as well as zebra mussel and therefore limited to use in contained systems (Claudi and Mackie 1994), or have not yet been registered for zebra mussel control. Although USEPA regulations may allow use of pesticide compounds in sites where they are registered (see next section), efficacy data should be consulted before any of these are used. Some pretreatment chemicals have been studied, such as the use of carbon dioxide before chlorination to narcotize the bivalves and cause them to "gape" (Elzinga and Butzlaff 1994).

Some of the proposed compounds are given in the following tabulation, along with their primary use.

Proprietary Compounds Proposed for Zebra Mussel Control but Not in General Use

Compound (Trade Name)	Chemical Name	Use	Reference
Clonitralid	5-chloro-n-(2-chloro-4-nitrophenyl) -2-hydroxybenzamide compound with 2-aminoethanol (1:1) (9ci)	Molluscicide Lampricide	
Niclosamide (Bayluscide 70)	2-aminoethanol salt of 2',5-dichloro-4',nitro-salicylanilide	Molluscicide Larvicide	
Bayer 73 (Bayluscide)	2',5-dichloro-4',nitro-salicylanilide	Lampricide	Fisher et al. 1994 Waller et al. 1993
Penaten®	Zinc oxide	Antifouling	Magee, Wright, and Setzler-Hamilton 1997
Rotenone (Noxfish)	1,2,12,12a-tetrahydro-2-iso-propenyl 8,9-dimethoxy-[1]bensopyrano-[3,4]furo [2,3-b] [1] benzo pyran-6 (6aH) one	Piscicide	Fisher et al. 1994
Salicylanilide I (Sal I)	2',5-dichloro-3-*tert*-butyl-6-methyl-4'-nitrosalicylanilide		Fisher at al. 1994 Waller et al. 1993
TFM (Lamprecid)	3-trifluoromethyl-4-nitrophenol	Lampricide	Fisher et al. 1994 Waller et al. 1993

A number of compounds derived from natural sources such as plant toxins have been tested for use in controlling zebra mussels (Taylor and Zheng 1995, 1997). In addition, antibiotic materials excreted by other aquatic organisms to keep them free of biofouling are relatively common, and these are being

investigated for their ability to prevent settling when applied as extracts or as a component in coatings. However, none of these has become commercially available yet. Compounds that are toxic to mussels are also potentially toxic to other life forms, and they must be tested and handled as carefully as other molluscicides.

One such natural compound, Endod, is a plant toxin product that includes chemicals called Lemmatoxins derived from the fruit of the African soap berry tree *Phytolacca dodecandra*. Two U.S. patents for its use as molluscicides have been awarded. Lemmatoxins have been shown to be lethal to zebra mussels at concentrations higher than 15 mg L^{-1}, while lower concentrations inhibited attachment and aggregation of adult mussels (Lemma et al. 1991; Lee, Lemma, and Bennett 1993). Toxicological studies have been done on nontarget mammals (Hietanen 1997).

USEPA Registration of Chemical Molluscicides

An understanding of the regulatory and legal standing of pesticide compounds such as molluscicides can be useful in handling these products. Howe et al. (1994) and Burns (1994) describe how FIFRA, enacted in 1972, relates to the registration and use restrictions of chemicals for zebra mussel control. The act monitors chemicals intended for control of living organisms and, as amended, requires registration and reregistration by the USEPA of pesticides sold or used in the United States to ensure that they will not cause unreasonable risk to the environment or human health when used according to the label directions. These regulations then apply to anyone who manufactures, formulates, markets, distributes, uses, or disposes of pesticide products, including aquatic biocides.

The primary registration mechanism is governed by FIFRA Section 3. Applications for registration of molluscicides may be for new active ingredients, the new use of a previously registered pesticide, or chemicals similar to currently registered compounds. The registration process (paid for by the registrant) is not inexpensive or fast because it requires detailed research by the registrant to determine the efficacy and environmental side effects of the active ingredient. Some of this testing is carried out via Experimental Use Permit (EUP) provisions under FIFRA Section 5. This may delay or prevent approval for use of the compound in a specific state. Most states require their own specific registration of pesticides in addition to registration with the USEPA. The expense of acquiring registration for biocidal compounds has understandably slowed the proposal and marketing of new chemicals specifically for the small area of zebra mussel control.

The effect of discharge of water containing molluscicidal chemicals on downstream receiving waters must be considered prior to the formulation of a treatment program. Even with discharge limits and requirements and the use of deactivation, there may be an effect on the ecosystem that needs to be avoided or restricted to certain times of the year (Claudi and Evans 1993). The legislation currently used to control direct discharges to waters of the Nation is the NPDES permit program. This was made possible by the passage of the Federal Water Pollution Control Act

Amendments of 1972 (also referred to as the Clean Water Act). These permits place limits on the amount of pollutants that may be discharged to waters by each discharger. These limits are set at levels protective of both the aquatic life in the waters that receive the discharge and human health. The Clean Water Act requires that registered biocides discharged to waters of the United States from a point source must be regulated such that WQBELs for that biocide are established in an NPDES permit to meet State water quality standards. There must also be compliance with each State's Antidegradation Policy. Thus, one of the label requirements for use of many aquatic biocides and pesticides in aquatic environments is to obtain an NPDES permit from the appropriate State/Tribal agency or USEPA Regional Office and to comply with State water quality requirements. Lack of a permit could result in enforcement action under FIFRA and the Clean Water Act. A risk-benefit analysis is also carried out by the USEPA, and a pesticide can be designated for "restricted use" if it is judged as presenting a high risk to humans or the environment. States usually require these chemicals to be applied only by certified applicators or people in their employ.

As well as FIFRA Section 3 registration, conditional use of pesticides may be authorized through Special Local Needs under Section 24(c); through Emergency Exemptions (ee) (Section 18); or through EUP provisions under Section 5. Use of a registered product on a pest not listed on the product label is allowed under Section 2(ee) as long as application is to a site stated on the label (Howe et al. 1994). However, specific registration for use in once-through cooling systems is required in many water handling operations where mussels are treated in these areas (Claudi and Mackie 1994).

It is important to remember that the product label of a registered pesticide is a legal document. Use of an aquatic biocide or molluscicide in a way that is inconsistent with the instructions provided on the label is a violation of FIFRA and can result in civil or even criminal action, via proceedings from the USEPA under FIFRA or from certain states (Howe et al. 1994). Compliance with the National Environmental Policy Act (NEPA) is required if Federal funds are used for zebra mussel control. This legislation dictates that control methods used at public facilities must not negatively affect native biota or existing water quality (Miller et al. 1992). A protocol for compliance with the NEPA process that should used in developing chemical control strategies for zebra mussel is described by Miller et al. (1992), and a working plan is reported on by Tippit, Cathey, and Swor (1993).

Sources of Additional Information

Dreissena! This newsletter is published six times per year by the National Zebra Mussel and Aquatic Nuisance Species Clearinghouse and presents the most current information and summaries of research, meetings, legislation, and sightings of zebra mussels (*http://www.entryway.com/seagrant/products.cfm#newsletters*). The Clearinghouse has a Web site at *http://www.entryway.com/seagrant/* .

Sea Grant Nonindigenous Species Site (SGNIS): *http://www.ansc.purdue.edu/sgnis/*

USGS Zebra Mussel Information Sources: *http://www.fcsc.gov/zebra.mussel/*

The U.S. Army Engineer Research and Development Center Zebra Mussel Research Program: *http://www.wes.army.mil/el/zebra/zebra.html*

The Zebra Mussel Information System (ZMIS) CD-ROM: *http://www.wes.army.mil/el/zebra/cd.html*

References

Allen, S. (1994). "Zebra mussel technical information report," Bulletin No. 11-494c. Calgon Corporation, Pittsburgh, PA. 4 p.

American National Standards Institute/National Sanitation Foundation. (1997). "Drinking water treatment chemicals - health effects," ANSI/NSF 60-1997, Ann Arbor, MI.

Barton, L. K. (1993). "Control program for zebra mussels (*Dreissena polymorpha)* at the Perry Nuclear Power Plant, Lake Erie." *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 555-562.

Blanck, C. A., Mead, D. F., and Adams, D. J. (1996). "Effective control of zebra mussels using a high molecular weight polymer." Abstract from the *Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 1996.

Burns, F. M. (1994). "Registration process required by the USEPA for a new pesticide," Technical Note ZMR-1-22, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Claudi, R., and Evans. D. W. (1993). "Chemical addition strategies for zebra mussel (*Dreissena polymorpha*) control in once-through service water systems." *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 563-573.

Claudi, R., and Mackie, G. L. (1994). *Practical manual for zebra mussel monitoring and control*. Lewis Publishers, Boca Raton, FL, 227 pp.

Dobbs, M. G., Cherry, D. S., Scott, J. C., and Petrille, J. C. (1995). "Environmental assessment of an alkyl dimethyl benzyl ammonium chloride (ADBAC) based molluscicide using laboratory tests." *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995. 87-101.

Electric Power Research Institute. (1993)."Hazard identification of commercially available biocides to control zebra mussels and Asiatic clams," TR-103175, Syracuse Research Corporation, Syracuse, NY.

Elzinga, W. J., and Butzlaff, T. S. (1994). "Carbon dioxide as a narcotizing pre-treatment for chemical control of *Dreissena polymorpha.*" *Proceedings, Fourth International Zebra Mussel Conference*, Madison, Wisconsin, March 1994. 45-59.

Fisher, S. W., Dabrowska, H., Waller, D. L., Babcock-Jackson, L., and Zhang, X. (1994). "Sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to candidate molluscicides," *J. Shellfish Res.* 13, 373-377.

Fisher, S. W., Stromber, P., Bruner, K. A., and Boulet, L. D. (1991). "Molluscicidal activity of potassium to the zebra mussel, *Dreissena polymorpha*: Toxicity and mode of action," *Aquatic Toxicology* 20, 219-234.

Green, R. F. (1995). "Strategies for application of non-oxidizing biocides," *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995. 175-181.

Hebert, P. D. N., Muncaster, B. W., and Mackie, G. L. (1989). "Ecological and genetic studies on *Dreissena polymorpha* (Pallas): A new mollusc in the Great Lakes," *Can. J. Fish Aquat. Sci.* 46, 1587-1591.

Heitanen, E. (1997). "Toxicity testing of Endod, a natural plant extract, as a prerequisite for its safe use as a molluscicide." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 549-563.

Howe, P. H., Masters, E., Atteberry, R., and Redmon, P. (1994). "A summary of Federal regulations related to use of FIFRA-registered biocides and Region 5, USEPA, use of these biocides for zebra mussel control," Technical Note ZMR-1-15, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Lawrence, D. F. (1997). "Chemicals for zebra mussel control," *Proceedings Seventh International Zebra Mussel and Aquatic Nuisance Species Conference*, New Orleans, January 28-31, 1997. 85-90.

Lee, H. H., Lemma, A., and Bennett, H. J. (1993). "The use of endod (*Phytolacca dodecandra*) to control the zebra mussel." *Zebra mussels: Biology, impacts and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 643-655.

Lemma, A., Wolde-Yohannes, L., Fraleigh, P. C., Klerks, P. L., and Lee, H. H. (1991). "Endod is lethal to zebra mussels and inhibits their attachment," *J. Shellfish Research* 10, 361-365.

McMahon, R. F., and Tsou, J. L. (1990). "Impact of European zebra mussel infestation to the electric power industry," *Annual Meeting, American Power Conference*, Chicago, IL, April 1990. 9 pp.

McMahon, R. F., Shipman, B. N., and Long, D. P. (1993). "Laboratory efficacies of nonoxidizing molluscicides on the zebra mussel (*Dreissena polymorpha*) and the Asian clam (*Corbicula fluminea*)." *Zebra mussels: Biology, impacts and control*. T. F. Nalepa and D.W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 575-598.

McMahon, R. F., Ussery, T. A., and Clarke, M. (1994). "Review of zebra mussel control methods," Technical Note ZMR-2-14, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Magee, J. A., Wright, D. A., and Setzler-Hamilton, E. M. (1997). "The use of Penaten to control zebra mussel attachment." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 541-548.

Miller, A. C., Cathey, H. J., Swor, T., and Tippit, R. (1992). "Developing a protocol to ensure environmental compliance in a zebra mussel control program," Technical Note ZMR-1-05, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Neilson, F. M. (1992). "Components of navigation locks and dams sensitive to zebra mussel infestations," Technical Note ZMR-3-08, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Netherland, M. D., and Getsinger, K. D. (1998). "Chemical control research strategy for zebra mussels," Technical Note ZMR-3-18, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Race, T. D., and Kelley, M. A. (1997). "A summary of a three year evaluation effort of anti-zebra mussel coatings and materials." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 359-388.

Taylor, G. T., and Zheng, D. (1995). "Natural products for mitigation of fouling by the blue mussel, *Mytilus edulis*, in marine water intake systems." *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995, 447-459.

Taylor, G. T., and Zheng, D. (1997). "Preliminary report on use of marine natural products as repellents for zebra mussels, *Dreissena polymorpha*," *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 523-531.

Tippit, R., Cathey, J., and Swor, T. (1993). "The NEPA process in a zebra mussel control program," Technical Note ZMR-1-10, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Van Benschoten, J. E., Jensen, J. N., Lewis, D., and Brady, T. J. (1993). "Chemical oxidants for controlling zebra mussels *(Dreissena polymorpha*): A synthesis of recent laboratory and field studies." *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 599-619.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). "Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms," *J. Great Lakes Res.* 19, 695-702.

2 Chlorination

The cost-effective oxidizing activity of chlorine has made it the most commonly used compound for all types of water disinfection and biofouling control in North America, and it has been used in potable water treatment since the beginning of the 1900's (Van Benschoten et al. 1993). Chlorination, primarily via sodium hypochlorite, has dominated the chemical control of zebra mussel in both Europe and North America, and remains the least expensive and most popular method of removal. Chlorination effects can be provided by a range of compounds—the hypochlorites of sodium, potassium, or calcium; chlorine and chlorine dioxide gases; and sodium chlorite—and their toxic properties can be used to control zebra mussels and related nuisance mollusk species. Chlorine is able to kill or prevent settling of planktonic veliger larvae in raw water piping systems. In general, chlorine treatment for zebra mussel control should be applied at the most suitable time, for the shortest period, and at the lowest concentration to be efficacious (Jenner and Janssen-Mommen 1993). However, adult mussels will close at concentrations of from 1 to 2 mg L^{-1} and remain closed for up to 2 weeks.

Claudi and Mackie (1994) discuss chlorination processes for zebra mussel control in industrial systems in detail, along with its pros and cons, and Netherland (1997) gives a brief summary of chlorine usage. Chlorine has a number of important advantages: it is relatively inexpensive, it works in most raw water systems, it is toxic at low concentrations and quickly loses toxicity without bioaccumulating, and it can be applied with simple mechanisms. However, there are several drawbacks to the chlorination process. The transport and storage of gaseous or liquefied chlorination products involve hazards, and their corrosive properties can harm system components, so that they all require special handling. Discharge also presents problems because carcinogenic compounds known as trihalomethanes may be formed where organic compounds are present in water. AOX (adsorbable organic halides) may also be formed, but do not present risks. Differences in water quality may incur extra costs where discharge concentrations may be unpredictable due to varying chlorine demand.

There has been concern that the cumulative effects of extensive chlorine use for zebra mussel control in large rivers in North America could be problematic due to toxicity to nontarget organisms and formation of trihalomethanes. Although chlorine discharge into natural water bodies is already regulated, it is possible that it may be prohibited or severely restricted in the future if usage increases significantly (Claudi and Mackie 1994). However, to date, these products provide the most effective and low-cost control in the majority of situations.

The next section reviews the general properties of chlorination via hypochlorite and chlorine gas; additional chlorine oxidizers (chlorine dioxide, sodium chlorite) are discussed separately.

Hypochlorite and Chlorine Gas

Chemical Names and Formulations

Chlorination compounds are available from numerous commercial sources as the following chemicals:

a. Calcium hypochlorite, $Ca(OCl)_2$; solid.

b. Sodium hypochlorite, NaOCl; liquid.

c. Potassium hypochlorite, KOCl.

d. Chlorine, Cl_2; gas.

Mode of Action

Chlorine controls zebra mussels through the effects of oxidation, consisting of either direct toxic effects on the adult, inhibition of settlement and growth of the larval stage, or weakening of the byssal thread attachments. Toxicity of chlorine to zebra mussels is a function of concentration, exposure time, and the type and quantity of chlorine compounds formed in water following treatment (Claudi and Mackie 1994).

Chlorine or hypochlorite reacts with water to form hypochlorous acid (HOCl), which readily dissociates to hydrogen ions (H^+) and hypochlorite (OCl^-). The hypochlorite ion is reduced to chloride ions and hydroxide ions forming a basic solution as it accepts electrons:

$$OCl^- + 2e^- + HOH \rightarrow Cl^- + 2\ OH^-$$

The ratio of hypochlorous acid to hypochlorite ions depends mainly on pH and to a lesser degree on temperature. Together, the two make up *free available chlorine* (FAC). The undissociated hypochlorous acid (HOCl) is a strong oxidizing agent and has the principal biocidal activity of these two chlorine species, damaging membranes, diffusing through cell walls, and disrupting enzyme activity, and perhaps affecting ion regulation (Claudi and Evans 1993; Claudi and Mackie 1994).

These FAC compounds react with ammonia and other nitrogen-containing compounds to make chloramines, which also contribute to disinfection and are known as combined available chlorine. These two types of available chlorines make up total residual chlorine (TRC) (Van Benschoten et al. 1993; Claude and Mackie 1994). The presence of organic nitrogen and other compounds reduces TRC because chlorine forms complex nitrogen compounds. Additional chlorine has to be added to obtain a specific TRC level, and this differential, called chlorine demand, varies with type of raw water and season.

Chlorination affects zebra mussels through toxic effects of free chlorine and chlorine products. It affects adults, inhibits settlement and growth of veligers, and weakens the byssal thread attachments that hold the mussels in place. Because mollusks sense chlorine at 0.04 mg L^{-1} total residual oxidant and close for extended periods to escape it, it is thought that oxidizing biocides can cause mussel mortality through asphyxiation or limited glycolysis over a prolonged period of constant chemical feed. In addition, the free chlorine and chlorine products have a chronic toxic effect. The toxic oxidant compound is also thought to accumulate as some siphoning goes on (Van Benschoten et al. 1993, 1995).

Application Strategies

End-of-season treatment. This treatment is given to flush out relatively small amounts of accumulated adults. Debris can be a problem; end-of-season (November) chlorination is not optimal if this is the only treatment or chemical being used.

Periodic treatment. This consists of at least three treatments of several weeks length; if given over the breeding season (May to November), adult mussels will be eliminated. Less debris is generated, as mussels are smaller.

Intermittent treatment. This treatment is useful for prevention of new primary veliger settlement, especially where adult mussels cannot be tolerated. However, it is not effective against established adults. It can be combined with an out-of-season continuous chlorination treatment to eliminate adults.

Semicontinuous. Frequent on/off cycling of treatment can have effects similar to continuous chlorination in keeping zebra mussels in a stressed status of shell closure. This treatment has a lower cost of material due to reduced exposure time.

Continuous. Constant presence of chlorine at low levels can prevent veliger settling and survival.

Chlorinating compounds can be added to water as gas (Cl_2), liquid (e.g., NaOCl), or solid (e.g., calcium hypochlorite, $Ca(OCl)_2$). Due to difficulty and hazard of handling pressurized chlorine gas, the majority of applications are of liquid hypochlorite. Various concentrations of sodium hypochlorite are available, with 12 percent active chlorine by weight being most commonly used by industry (Claudi and Mackie 1994). Chlorine gas requires potable water supply for maintenance and operation of a gaseous chlorine system, and air scrubbers to filter exhaust.

Timing of Application

If a single long-term application is being used to kill all mollusks present, it should be made following the reproduction period to ensure that no additional veliger settling will occur. Reproduction in zebra mussel is also dependent on water temperature, and this timing will be keyed in to the time of year.

A one-time application of this kind can be done if infestation is low enough that killed material will flush out without blocking the system. If infestation is greater and may block any parts of the system, twice-a-year chlorination may be required.

Winter treatment with low levels of chlorine may be useful for ridding systems of adult zebra mussels, although the process is slow (Van Benschoten et al. 1993).

For veliger control there is no need to chlorinate during winter when temperatures are too low for mussel reproduction, and chlorination can be suspended during periods when veligers are not present in intake water (Payne and Lowther 1992; Claudi and Mackie 1994)

Application Rates

Concentration and exposure time. Control of zebra mussel depends upon chlorine concentration, contact time, and water quality and temperature, where the relationship between concentration and exposure time is usually an inverse one. Van Benschoten et al. (1993, 1995) give models for predicting 95 percent mortality as a function of temperature and total residual chlorine concentration. Generally, the contact time required decreases as concentration increases; however, reducing chlorine concentrations by half results in half the mortality in less than twice the contact time, and this may be a way to reduce chemical costs (Van Benschoten et al. 1993). Water temperature is an important factor in effectiveness of chlorination in zebra mussel control; since chlorination is usually held at ambient temperature at treatment sites, the seasonal timing of chlorine application is important (Claudi and Mackie 1994). Higher concentrations of chlorine are required at lower temperatures in order to be equally effective. Chlorination may not be practical at less than 50 ºF (10 ºC), due to the longer contact time required.

Chlorine demand. Chlorine demand of incoming water should be established and included in calculating the application concentration. A guide to these calculations is given in Claudi and Mackie (1994). Consider effects of pH, organic and inorganic nitrogen content, temperature, and physiological status of zebra mussels on concentrations required for effective treatment. Water with large amounts of organic and inorganic compounds has high chlorine demand. Most of this information given here comes from experience with Great Lakes water; results may differ with other water qualities (Claudi and Evans 1993).

Mortality. Generally, 0.5 mg chlorine L^{-1} (ppm) for 2 hr gave 100 percent mortality in veligers (Klerks, Fraleigh, and Stevenson 1993). Fatality in adult mussels occurred at concentrations of 2.0 mg L^{-1}.

End-of-season treatment. While chlorination is not the most efficient method where a single annual application is used to eliminate established adult mussels, it can be used in this way by applying high doses continuously for 2 to 3 weeks. Results from tests in the field (Claudi and Mackie 1994) are shown in the following tabulation.

Mortality with End-of-Season Chlorine Treatment		
Chlorine Concentration mg L^{-1}	**Exposure Time weeks**	**Mortality percent**
0.5	2	95
1.0	9	100
1.5	7	100
2.0	3	90
2.0	6	100

Periodic. Similar results will be produced by the treatment levels used in single end-of-season applications (Claudi and Mackie 1994). Other research (McMahan and Tsou 1990; Jenner and Janssen-Mommen 1993) is summarized in the following tabulation.

Mortality with Periodic Chlorine Treatment		
Concentration mg L^{-1}	**Exposure Time days**	**Results % kill**
0.5	7	75
0.3	>14 to 21	> 95
0.5 TRC or 0.5 TRO[1]	14 to 21 at 20 to 25 ºC (68 to 77 ºF)	> 95
[1] Total residual oxidant.		

Intermittent. Intermittent treatment, used throughout the breeding and settlement period at rates of 2 mg L^{-1} chlorine (TRC) for half-hour periods at 12-hr intervals, has been effective in preventing primary veliger settlement but not in removing established adults (Claudi and Mackie 1994). Such a treatment can be combined with an out-of-season continuous chlorination treatment for 2 to 6 weeks at 2 mg L^{-1} TRC to eliminate accumulated adults (Claudi and Evans 1993). Treatment combinations are shown in the following tabulation.

Mortality with Various Concentration, Exposure, and Interval Combinations of Intermittent Treatments				
Concentration	**Exposure min**	**Interval hours**	**Mortality**	**Reference**
0.8 % 0.5 mg L^{-1} TRC	30	12	100 % veliger No settlement	Barton 1993
2 mg L^{-1}	30	12	No new veliger settlement	Claudi and Evans 1993

Semicontinuous. On/off cycling of chlorine over short intervals takes advantage of the lag time of mussel shell opening after treatment is discontinued to mimic continuous treatment while reducing total residual oxidant loading significantly. It is expected to be comparable to continuous treatment in effects, but with lower chemical use and discharge, and subsequently lower cost. Tests showed that a cycle of 15 minutes of 0.5 ppm chlorine followed by 30 minutes without chlorine reduced settling to levels found with continuous treatment (Claudi and Mackie 1994). Results are summarized in the following tabulation.

Mortality with Semicontinuous Cycling		
On/Off Cycle min	**TRC Concentration**	**Live Mussels on Surface**
15/15	0.5	12
15/30	0.5	2
15/15	0.3	64
Untreated Control	0	4,993

Continuous. Low, sublethal levels of chlorination may be effective against zebra mussel by eventually producing chronic toxicity or preventing macrofouling. Speed of water flow may affect how readily mussels are detached and swept away. Continuous treatment at 0.5 mg L^{-1} TRC has been successful, preventing new settlement and killing adult mussels regardless of speed of flow (Claudi and Mackie 1994). The following tabulation shows several rates.

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Mortality with Continuous Treatment			
Concentration mg L^{-1}	**Exposure days**	**Results**	**References**
0.5	90	100% Prevented all new settlement	Claudi and Evans 1993
0.3	90	100% Prevented all new settlement	Claudi and Evans 1993
0.3 to 0.5	14 to 21	100%	Miller, Payne, McMahon 1992

Maximum Water Concentration

Discharge limits for specific facilities depend on local, State and Federal water restrictions as permitted under the National Pollutant Discharge Elimination System program.

For power plants greater than 25 MW, the U.S. Environmental Protection Agency effluent limitation guideline for chlorine is 0.2 mg L^{-1} (ppm) TRC (Federal Register, 40 CFR Part 423, November 1982). This discharge concentration is limited to 2 hr per day unless the need for use in combating macrofouling is demonstrated (Jenner and Janssen-Mommen 1993).

Other discharge configurations may be permitted. For example, the Perry Nuclear Power Plant, North Perry, Ohio, has a regulatory discharge limit for chlorine of maximum daily discharge time of 2 hr, with concentration limits of 0.2 mg L^{-1} for a 30-day average and 0.5 mg L^{-1} on a daily basis (Barton 1993).

Use Restrictions

Production of trihalomethanes must not exceed 80 ppb (Fg/L) in drinking water (U.S. Environmental Protection Agency 1994). Prior to this, the standard was 100 ppb.

Timing and Appearance of Effects

Considerable lag times between application and adult zebra mussel death have been observed, presumably due to shell closure when the presence of oxidant is sensed. Lag times of from 2 to 18 days are noted and generally decrease as chlorine concentration increases (Van Benschoten et al. 1993).

Resistance to chlorine can vary with age, size, and developmental stage of the mussel, with older and larger individuals being more resistant; veligers are much more susceptible than adults (Claudi and Mackie 1994).

Toxicological Data

Toxicology of sodium hypochlorite for two important aquatic species is shown in the following tabulation:

Aquatic Toxicology of Sodium Hypochlorite (NaOCl): 5% Active Chlorine Solution[1]

Species	Test[2]	Concentration mg L^{-1}
Rainbow trout	48-hr LC_{50}	0.07
Fathead minnow	96-hr LC_{50}	5.9

[1] From Materials Safety and Data sheet (ACROS Organics 1996).
[2] Concentration lethal to 50 percent of the individuals.

Sodium hypochlorite is broken down in the environment into sodium chloride, oxygen, and water. Other substances may be formed to a limited extent. These by-products are often referred to as AOX (adsorbable organic halides). A great many studies have been made to provide a risk assessment of NaOCl in terms of its formation of AOX. It was concluded that the amount of AOX is very small both in absolute terms and relative to other human activities and natural sources. The majority of these compounds are easily degradable and are primarily water soluble and not bioaccumulative. Highly chlorinated species, such as dioxins, are not formed.

Precautions

Possible by-products from antifouling chlorination may include chlorobromoform, halogenated benzenes, and phenols (Jenner and Janssen-Mommen 1993).

Sodium hypochlorite is corrosive and causes burns to eyes, skin, and internal organs if ingested or inhaled.

When handling sodium hypochlorite, wear rubber gloves and splash shield.

Chlorine gas presents risk of potentially dangerous leaks. Extensive safety training may be required for those handling the material and for any response team.

Field Instructions and Guidance on Operational Applications

Store sodium hypochlorite solution away from heat and light to prevent decomposition, such as in polyethylene tanks vented to release oxygen. Provide containment basins. Avoid use of stainless steel in storage or handling. If dilution is required prior to treatment, use only deionized or distilled water.

The standard industrial strength 12 percent solution of sodium hypochlorite can precipitate calcium carbonate ($CaCO_3$) where raw water contains high levels of calcium. Allow for this by avoiding small-diameter piping, etc.

To monitor residual chlorine, most agencies and industries analyze for chlorine using automatic on-line or laboratory testing based on the amperometric (electrochemical) titration method with detection in the range of 2 to 5 ppb. However, a number of compounds regularly present in water can interfere with detection. Colorimetric (spectrophotometric) and potentiometric (electrode-based) analyses are also available. Claudi and Mackie (1994) provide details and further references.

Adjuvant Use or Deactivation/Detoxification

Dilution is the most common means of detoxification of treated water.

The use of activated carbon filters allows removal of chlorine without replacement with another salt, and this is the most effective means of actual dechlorination (Menis-Croxall and deBruyn 1997).

Where chlorine dosage is at high concentrations or relatively frequent, dechlorination may be required to meet discharge regulations, unless outflow is to a storage lagoon or is diluted to acceptable levels. Dechlorination can be done by addition of sodium sulfite (Na_2SO_3), sodium bisulfite ($NaHSO_3$), sodium metabisulfite ($Na_2S_2O_5$), or sulfur dioxide (SO_2) (Barton 1993; Claudi and Mackie 1994). Sodium bisulfite in solution, known as liquid sulfite, is commonly used at 1.8 to 2.0 mg L^{-1} of sulfite per mg L^{-1} of residual chlorine, and can be fed into the discharge waters at any time as it acts rapidly (Claudi and Mackie 1994).

Application Techniques

Use of sodium hypochlorite solution. Apply at a single point where it will be able to be well-mixed into system water. Use accurate metering pumps or flowmeters engineered to handle the highly corrosive and alkaline properties of the solution. Diaphragm pumps are recommended, either motor or electric solenoid driven. Teflon fittings are required, and fiberglass piping is recommended; avoid stainless steel. Specific considerations for the holding tank, containment area, metering pump systems, and the skids to hold them are discussed by Menis-Croxall and deBruyn (1997). Use fiberglass piping for transporting concentrated solution to point of application.

Use of chlorine (Cl_2) gas. Injection of the gas is more hazardous due to the nature of the material.

Antidote Information

The following antidotes are from Materials Safety and Data Sheet for 5 percent sodium hypochlorite (ACROS Organics 1996):

a. Eyes. Flush eyes thoroughly with plenty of water for at least 15 minutes. Get medical aid immediately.

b. Skin. Flush with plenty of soap and water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid immediately.

c. Ingestion. Do not induce vomiting. Get medical aid immediately. Wash mouth out with water.

d. Inhalation. Get medical aid immediately. Remove the victim from exposure to fresh air immediately. If the victim is not breathing, give artificial respiration. If the victim's breathing is difficult, give oxygen.

References

ACROS Organics. (1996). "Material Safety Data Sheet: Sodium hypochlorite (5% chlorine), reagent." Fairlawn, NJ.

Barton, L. K. (1993). "Control program for zebra mussels (*Dreissena polymorpha)* at the Perry Nuclear Power Plant, Lake Erie." *Zebra mussels: Biology, impacts, and control.* T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 555-562.

Claudi, R., and Evans. D. W. (1993). "Chemical addition strategies for zebra mussel (*Dreissena polymorpha*) control in once-through service water systems." *Zebra mussels: Biology, impacts, and control.* T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 563-573.

Claudi, R., and Mackie, G.L. (1994). *Practical manual for zebra mussel monitoring and control.* Lewis Publishers, Boca Raton, FL, 227 pp.

Federal Register. (1982). "Steam electric power generating category," 40 CFR Part 423.

Jenner, H. A., and Janssen-Mommen, J. P. M. (1993). "Monitoring and control of *Dreissena polymorpha* and other macrofouling bivalves in the Netherlands." *Zebra mussels: Biology, impacts, and control.* T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 537-554.

Klerks, P. L., Fraleigh, P. C., and Stevenson, R. C. (1993). "Controlling zebra mussel (*Dreissena polymorpha*) veligers with three oxidizing chemicals: Chlorine, permanganate, and peroxide + iron." *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 621-641.

McMahon, R. F., and Tsou, J. L. (1990). "Impact of European zebra mussel infestation to the electric power industry." *Annual Meeting, American Power Conference*, Chicago, IL, April 1990. 9 pp.

Menis-Croxall, N., and deBruyn, G. (1997). “Zebra mussel mitigation: Elements of a successful chlorination system.” *Proceedings, Seventh International Zebra Mussel and Aquatic Nuisance Species Conference*, New Orleans, January 28-31, 1997. 97-105.

Miller, A. C., Payne, B. S., and McMahon, R. F. (1992). “Zebra mussels: Biology, ecology, and recommended control strategies,” Technical Note ZMR-1-01, Zebra Mussel Research Program. U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS, 10 pp.

Netherland, M. D. (1997). “Chemical control research strategy for zebra mussels,” Technical Note ZMR-3-18, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS, 7 pp.

Payne, B. S., and Lowther, D. (1992). “Larval monitoring in a chlorine treatment program to prevent zebra mussel settlement in hydropower facilities,” Technical Note ZMR-1-02, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS. 2 pp.

U.S. Environmental Protection Agency. (1994). “National primary drinking water regulations; disinfectants and disinfection byproducts; proposed rule.” *Fed. Regist.* 59, 38668-38829.

Van Benschoten, J. E., Jensen, J. N., Lewish, D., and Brady, T. J. (1993). “Chemical oxidants for controlling zebra mussels *(Dreissena polymorpha*): A synthesis of recent laboratory and field studies.” *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 599-619.

Van Benschoten, J. E., Jensen, J. N., Harrington, D., and DeGirolamo, D. J. (1995). “Zebra mussel mortality with chlorine,” *J. Amer. Water Works Assoc.* 87, 101-108.

Chlorine Dioxide

Compared with chlorination with sodium hypochlorite, chlorine dioxide has several advantages. It is efficacious at lower concentrations; does not produce trihalomethanes (its by-products are sodium chloride and sodium chlorite); is not affected by pH or ammonia; and requires only short treatment duration and thus has less effect on system operations (Tsou et al. 1995). Some reports suggest that chlorine dioxide may be more effective than chlorine against adult mussels (Rusznak, Mincar, and Smolik 1994; Garrett and Laylor 1995; Matisoff, Brooks, and Bourland 1996). It has been used as a disinfectant for water for several decades. Disadvantages include the requirement for onsite generating equipment; storage of the component precursor chemicals (sodium hypochlorite and hypochloric acid); high oxidant demand, which may require higher treatment rates and reduce efficacy on the mussels; and conversion of the dioxide to chlorite, which limits the amount of ClO_2 that can be applied without excessive chlorite discharge.

Chemical Name and Formulations

Chlorine dioxide, ClO_2, is a yellow-green water-soluble gas. The generic chemical is available from numerous commercial sources.

Mode of Action

The gas is a biotoxic oxidant, causing membrane damage.

Application Strategies

Application is by injection of chlorine dioxide gas manufactured onsite using temporary or permanently installed generation and detoxification equipment. Chlorine dioxide has been applied in operational trials using a GENEROX™ generator from a ZEBRA MUSSEL BUSTER™ trailer (Holt and Ryan 1997).

Application can be on- or off-line.

Timing of Application

Application may be one of the following:

a. *Periodic*: approximately three times per year for 2 to 4 days at each time, to intake water. Can be used to perform periodic adult eradication (Tsou et al. 1995; Holt and Ryan 1997).

b. *Intermittent*: low-level feed of chlorine dioxide will control microbiological growth and prevent settlement of postveligers.

c. *Continuous or semicontinuous*: using permanently installed ClO_2 generation and detoxification equipment

Application Rates

The application may be at one of the following rates:

a. Continuous:

(1) Prevent veliger settlement: 0.125 mg L^{-1} ClO_2 (Klerks, Fraleigh, and Stevenson 1993).

(2) Veliger control: 0.25 to 5.0 mg L^{-1} for 3 to 9 days (Rusznak et al. 1995).

(3) Adult control: 0.15 to 5.0 mg L^{-1} above oxidant demand for 2 to 4 days (Smolik et al. 1995).

b. Intermittent: 0.25 mg L^{-1} for 15 minutes duration, four times daily, reduced settlement by 95 percent versus an untreated system and successfully controlled postveliger mean densities to less than 600 individuals per m^2 (Mallen et al. 1997).

c. Periodic: 2- to 4-day applications injected at 0.6 to 1 ppm, 3 to 4 times a year gave 70 to 100 percent mortality of adults in bioboxes (Tsou et al. 1995; Holt and Ryan 1997).

Maximum Water Concentration

Discharge limits depend on local, State, and Federal water restrictions as permitted under the National Pollutant Discharge Elimination System program.

Use Restrictions

Restrictions involve maximum contaminant levels on trihalomethanes, as well as on the sum of residual chlorine dioxide, chlorite, and chlorate (Van Benschoten et al. 1993).

Timing of Results

Considerable lag times between application and adult zebra mussel death have been observed, presumably due to shell closure when the presence of oxidant is sensed. Lag times of from 2 to 18 days are noted and generally decrease as chlorine concentration increases (Van Beschoten et al. 1993).

Resistance to chlorination can vary with age, size, and developmental stage of the mussel, with older and larger individuals being more resistant; veligers are much more susceptible than adults (Claudi and Mackie 1994).

Toxicological Data

For precursor sodium hypochlorite, see preceding section on hypochlorite.

Precautions

Chlorine dioxide has the following hazards and requires the following precautions:

a. Corrosive, severe respiratory and eye irritant.

b. May explosively decompose on shock, friction, concussion, or rapid heating.

c. Strong oxidant--reacts violently with combustible and reducing materials, and with mercury, ammonia, sulphur, and many organic compounds.

d. Safety glasses, face shield, gloves. Use effective ventilation.

e. In dilute solution, is explosive at concentrations over 10 percent.

Field Instructions and Guidance on Operational Applications

Wear splash-proof chemical goggles when working with liquid, unless full-face-piece respiratory protection is worn.

Wear dustproof goggles when there is a potential for exposure to the gas, unless full-face-piece respiratory protection is worn.

Adjuvant/Detoxicant/Deactivant Use

Residual chlorine has been neutralized with sodium bisulfite prior to discharge, at 7 ppm dechlorinating agent for 1 ppm oxidant (Tsou et al. 1995).

Application Techniques

Various mobile or onsite generation and application systems for sodium chlorite are available. These usually generate the gas from a combination of sodium hypochlorite, sodium chlorite, and hydrochloric acid.

The “vapor-phase” gaseous chlorine/liquid chlorite generation system is patented by Rio Linda (Rybarik, Byron, and Germer 1995).

The Drew CLO_2 generator reacts a sodium chlorite solution with sodium hypochlorite and hydrochloric acid (Tsou et al. 1995).

Once generated, the gaseous chloride dioxide is mixed with water and pumped to location of use.

Antidote Information

Use the following antidotes and first-aid procedures:

a. *Eyes*: Flush eyes thoroughly with plenty of water for at least 15 minutes. Get medical aid immediately.

b. *Skin*: Flush with plenty of soap and water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid immediately.

c. *Ingestion*: Do not induce vomiting. Get medical aid immediately. Wash mouth out with water.

d. *Inhalation*: Get medical aid immediately. Remove victim from exposure to fresh air immediately. If victim is not breathing, give artificial respiration. If victim's breathing is difficult, give oxygen.

References

Claudi, R., and Mackie, G. L. (1994). *Practical manual for zebra mussel monitoring and control.* Lewis Publishers, Boca Raton, FL, 227 pp.

Garrett, W. E., Jr., and Laylor, M. M. (1995). "The effects of low level chlorination and chlorine dioxide on biofouling in a once-through service water system." *Proceedings Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference,* Toronto, Canada, February 1995. pp 135-149.

Holt, D. A., and Ryan, E. (1997). "Chlorine dioxide--adult zebra mussel eradication at the Dofasco Steel Mill." *Proceedings Seventh International Zebra Mussel and Aquatic Nuisance Species Conference,* New Orleans, January 28-31, 1997. 59-67.

Klerks, P. L., Fraleigh, P. C., and Stevenson, R. C. (1993). "Controlling zebra mussel (*Dreissena polymorpha*) veligers with three oxidizing chemicals: Chlorine, permanganate, and peroxide + iron." *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 621-641.

Matisoff, G., Brooks, G., and Bourland, B. I. (1996). "Toxicity of chlorine dioxide to adult zebra mussels," *J. Amer. Wat. Works Assoc.* 88, 93.

Mallen, E., Freymark, S., Hale, L., and Lippincott, B. (1997). "Use of chlorine dioxide to prevent zebra mussel settlement." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 489-499.

Rusznak, L. H., Mincar, G. J., and Smolik, N. A. (1994). "Chlorine dioxide--a molluscicidal agent for adult zebra mussel eradication." *Proceedings Fourth International Zebra Mussel Conference,* Madison, WI, March 1994. 127-137.

Rusznak, L., Smolik, N., Hale, L., and Freymark, S. (1995). "Examination of the potential of chlorine dioxide for use in zebra mussel veliger control." *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995. 371-377.

Rybarik, D., Byron, J., and Germer, M. (1995). “Chlorine dioxide treatment for zebra mussel control.” *Proceedings Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995. 379-398.

Smolik, N., Rusznak, L., Anderson, J., and Hale, L. (1995). “The use of chlorine dioxide for zebra mussel control--a perspective of treatment histories.” *Proceedings Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 1995. 405-417.

Tsou, J., Coyle, J., Pall, S., Mussalli, Y., and Martin, P. (1995). “Evaluation of the use of chlorine dioxide to control zebra mussels.” *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada. 461-473.

Van Benschoten, J. E., Jensen, J. N., Lewish, D., and Brady, T. J. (1993). “Chemical oxidants for controlling zebra mussels *(Dreissena polymorpha*): A synthesis of recent laboratory and field studies.” *Zebra mussels: Biology, impacts, and control*. T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 599-619.

3 Nonchlorine Oxidizing Chemicals

In addition to the chlorinating chemicals, the oxidizers bromine and potassium permanganate can be used for treatment of zebra mussels.

Bromine

Bromine is an oxidizing chemical that is used for antifouling purposes in such forms as activated bromine, sodium bromide, bromine chloride, and mixtures of bromine and chlorine or other chemicals (Claudi and Mackie 1994), and it is approved for use in the United States for zebra mussel control. It is more effective as an oxidizing agent when water pH is above 8.0 (Fellers, Flock, and Conley 1988). Bromine is also used as a chlorine enhancer designed to minimize the amount of chlorine required to prevent macrofouling, and the proprietary ACTI-BROM® compounds are used in this way. BROMICIDE® and LIQUIBROM™ are other examples of bromine, marketed by Great Lakes Chemical Corporation, West Lafayette, IN.

In the past it was suggested that bromine was less toxic than chlorine to nontarget species, but this has been shown not to be the case (Howe et al. 1994). Total amount of oxidant required for mussel control is approximately the same as chlorine (Claudi and Mackie 1994).

ACTI-BROM

ACTI-BROM® is a chlorine enhancer system that consists of an aqueous solution containing a bromide salt and an oxyalkylate biodispersant (surfactant) designed to improve chlorine activity. The ACTI-BROM technology was originally patented for control of the Asiatic clam and barnacles, and the application program has been found to be effective on zebra mussels (McCarthy and Trulear 1992). It can be particularly useful in situations where control cannot be obtained within the legal chlorination limits or where sodium hypochlorite is being considered as a gaseous chlorine alternative. This compound can be used in either eradication or prevention treatment programs.

Chemical Name and Commercial Formulations

This molluscicide is characterized as follows:

a. *Chemical name*: Sodium bromide, $NaBr_2$.

b. *Formulation*: ACTI-BROM 1338 Biodispersant.

- 42.8 percent sodium bromide plus an oxyalkylate.
- Aqueous solution.

c. *Source*: Nalco Chemical Company
One Nalco Center
Naperville, IL 60563-1198
(630) 305-1000
Emergencies: 1-800-462-5378

Mode of Action

ACTI-BROM is an oxidizing biocide program that utilizes a chlorine source, either gas or sodium hypochlorite, to activate a bromide/biodispersant chemistry. On their own, the bromide salt and biodispersant present in ACTI-BROM are nonmolluscicidal; however, in the presence of chlorine gas or sodium hypochlorite in situ, ACTI-BROM's bromine portion is activated to form hypobromous acid and hypobromite ion, depending on water pH (McCarthy and Trulear 1992). Both these forms of bromine are lethal to zebra mussels, destroying vital tissue. Hypochlorous acids are also formed, and the ratio of hypobromous to hypochlorous acid can be varied by altering the molar ratios between sodium bromide-surfactant and the chlorine source.

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

or

$$NaOCl + H_2O \rightarrow HOCl + NaOH$$

then

$$HOCl + NaBr \rightarrow HOBr + NaCl$$

The oxyalkylate surfactant, present at 5 to 10 ppb, is not expected to contribute to toxicity, but may inhibit settling and attachment by acting as a biodispersant.

Application Strategies

Add sufficient ACTI-BROM and oxidize with either chlorine gas or sodium hypochlorite solution to achieve residual bromine levels as needed.

Two primary types of treatment programs are recommended for controlling zebra mussels with this product:

a. *Eradication*: eliminate accumulated mussels.

b. *Preventive*: prevent migration and attachment of zebra mussels in a system.

Timing of Application

Application is timed according to the following treatments:

a. *Periodic*: usually applied twice per year.

b. *Eradication*: apply one to three times per year during warm season: immediately after the annual peak in reproduction (typically in June or July), and at the end of the season (October or November) (Nalco Chemical Company 1996).

c. *Preventive*: treat throughout warm weather season. Can be added whenever chlorination is applied.

Application Rates

Add sufficient ACTI-BROM 1338 and oxidize with either gas chlorine or sodium hypochlorite solution to achieve a reisdual bromine level of 0.5 to 5 ppm or as needed to maintain control of the system.

Periodically apply on a continuous basis for 1 to 3 weeks at 0.1 to 0.5 ppm free residual oxidant to eradicate juvenile and adult mussels (McCarthy and Trulear 1992). This concentration is based on a bromine to chlorine ratio (molar basis) of 1:1.

Treatment of adults with a total residual oxidant level of 0.1 ppm indicated that 18-day continuous contact gave up to 60 percent mortality, and 30-day contact 90 to 100 percent mortality (Sawyko 1994).

Maximum Water Concentration

This product is registered as a pesticide by the U.S. Environmental Protection Agency and must be handled and fed within the limits of the label instructions.

Use Restrictions

Use with a registered gaseous chlorine or sodium hypochlorite product.

Apply in accordance with the label and with the site National Pollutant Discharge Elimination System permit.

For zebra mussel control in New York State, the Department of Environmental Conservation provides a “generic modification” for holders of State Pollutant Discharge Elimination System permits to allow bromine treatments, including ACTI-BROM.

Timing and Appearance of Effects

Effect is synchronous with chlorine cotreatment effect. It produces more rapid effects in veliger stages than in adults.

Toxicological Data

The Signal Word is Caution.

Toxicological data for bromine to aquatic species is shown in the following tabulation:

Aquatic Toxicology Data for Various Forms of Bromine[1]

Organism	Test	Data[2]
ACTI-BROM		
Rainbow trout	96-hr Static	LC_{50} > 1,000 mg/L NOEL 1,000 ppm
Bluegill sunfish	96-hr Static	LC_{50} > 1,000 mg/L NOEL 1,000 ppm
Sodium Bromide		
Fathead minnow	96-hr Static	LC_{50} 16,479 mg/L
Poecilia reticulata	96-hr Static	LC_{50} 225 mg/L
Daphnia magna	48-hr Static	LC_{50} 7,900 mg/L
Hypobromous Acid (from Product)		
Fathead minnow	96-hr Flow-through	LC_{50} 0.079 mg/L (as Br_2)
Daphnia magna	48-hr Flow-through	LC_{50} 0.038 mg/L (as Br_2)
Hypobromous Acid (from Sodium Bromide)		
Bluegill sunfish	96-hr Static	LC_{50} 0.52 mg/L (as Br_2) NOEC 0.30 ppm (as Br_2)
Rainbow trout	96-hr Static	LC_{50} 0.23 mg/L (as Br_2)
Fathead minnow	96-hr Flow-through	LC_{50} 0.097 mg/L (as Br_2)
Sheepshead minnow	96-hr Flow-through	LC_{50} 0.19 mg/L (as Br_2) NOEC 0.08 ppm
Daphnia magna	48-hr Static 48-hr Flow-through	LC_{50} 0.71 mg/L (as Br_2) NOEC 0.41 ppm LC_{50} 0.038 mg/L (as Br_2)
Eastern oysters	96-hr Flow-through	EC_{50} 0.54 mg/L (as Br_2)
Mysid shrimp	96-hr Flow-through	LC_{50} 0.54 mg/L (as Br_2)

[1] From Materials Safety and Data Sheet (Nalco Chemical Company 1992); see also Electric Power Research Institute (1993).
[2] LC_{50} = Median lethal concentration
NOEL = No-observed-effect level
NOEC = No-observed-effect concentration

Precautions

ACTI-BROM is harmful if swallowed and causes moderate eye irritation. Do not get in eyes, on skin, or on clothing.

Field Instructions and Guidance on Operational Applications

Use impermeable gloves and chemical splash goggles when handling.

Although ACTI-BROM produced mortality below 20 °C (68 °F), lower water temperatures may reduce the effectiveness of treatment (Sawyko 1994).

Monitoring kits for use in checking zebra mussel veliger density and settling in a water system and experimental systems for determining adult mussel mortality under local water quality conditions are available from Nalco.

Adjuvant and Deactivation Use

Use ACTI-BROM as an adjuvant to chlorination treatment.

A 60 percent potassium hypochlorite (solid) is used for weekly addition to supplement bromine treatment (Schnelle and Strimple 1995).

It may be necessary to use a dehalogenating material such as a sodium bisulfite to reduce halogen levels prior to discharge, depending on outfall permits.

Application Techniques

ACTI-BROM can be added whenever chlorination is applied. It can be fed with two pumps, one for sodium hypochlorite and one for sodium bromide solution, directly from the drum or bulk storage tank to a location in the chlorination system where it will be uniformly mixed and thoroughly distributed.

Feed as far upstream as possible.

Monitor treatment levels of ACTI-BROM and oxidant with test kits for bromine or chlorine.

Antidote Information

Use the following antidote and first aid procedures:

a. *Eyes*: flush with water for 15 minutes. Call a physician.

b. *Skin*: wash thoroughly with soap and rinse with water. Call a physician.

c. *Ingestion*: induce vomiting and give water, except when the victim is unconscious, having trouble breathing, or in convulsions. Call a physician.

d. *Inhalation*: remove victim to fresh air. Treat symptoms. Call a physician.

e. *Emergencies*: 1-800-462-5378 (Nalco, 24 hour-a-day response).

References

Claudi, R., and Mackie, G. L. (1994). *Practical manual for zebra mussel monitoring and control.* Lewis Publishers, Boca Raton, FL, 227 pp.

Electric Power Research Institute. (1993).“Hazard identification of commercially available biocides to control zebra mussels and Asiatic clams,” TR-103175, Syracuse Research Corporation, Syracuse, NY.

Fellers, B. D., Flock, E. L., and Conley, J. C. (1988). “Bromine replaces chlorine in cooling-water treatment,” *Power* 132(6), 15-20.

Howe, P. H., Masters, E., Atteberry, R., and Redmon, P. (1994). “A summary of Federal regulations related to use of FIFRA-registered biocides and Region 5, USEPA, use of these biocides for zebra mussel control,” Technical Note ZMR-1-15, Zebra Mussel Research Program, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

McCarthy, R. E., and Trulear, M. G. (1992). “Efficacy of an oxidizing molluscicide against zebra mussels: Result of laboratory and field studies.” *Proceedings of Corrosion ‘92, NACE [National Association of Corrosion Engineers] International*, Nashville, TN, May 1992. Houston, TX, 5 pp.

Nalco Chemical Company. (1992). “Material Safety Data Sheet: ACTI-BROM 1338 Biodispersant,” Naperville, IL, 8 pp.

Nalco Chemical Company. (1996). “Control of zebra mussels: Program profile,” Naperville, IL, 5 pp.

Sawyko, P. M. (1994). “Development of zebra mussel control programs utilizing chlorine, ACTI-BROM, and bromine.” *Proceedings, Fourth International Zebra Mussel Conference*, Madison, WI, March 1994, 139-148.

Schnelle, R. C., and Strimple, P. D. (1995). “A preliminary study on the effects of combined halogenation and thermal recirculation at a coal-fired generating station.” *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 14-21, 1995. The Professional Edge, Pembroke, Ontario, Canada, 399-403.

Potassium Permanganate

Potassium permanganate, a purplish chemical whose use as a disinfectant was originally developed in the 1800's, is a strong nonchlorine oxidant with a long history of safe use in drinking water, wastewater, and chemical manufacturing industries. It has been used by water treatment plants as an oxidizer since the turn of the century, and is commonly used in municipal facilities for water purification. It is widely used for oxidation of iron and manganese and to correct taste and odor problems in treated water because of its ability to produce oxidation reactions with inorganic compounds and organic substances (Claudi and Mackie 1994).

Potassium permanganate produces effective control of adult zebra mussel at 2.0 mg L^{-1}, and inhibits veliger settlement at 1.0 mg L^{-1} and below (San Giacomo and Wymer 1997). It does not produce trihalomethanes or haloacetic acids (San Giacomo and Wymer 1997). Although it costs more than chlorine, it can be less expensive than proprietary molluscicide chemicals. It requires a long contact time. It may not be 100 percent effective (Fraleigh et al. 1993). Overdosing may result in an unacceptable pink coloration in water. Agencies using this control method include the City of Baltimore, Public Works (Balog et al. 1995) and the City of Buffalo, NY (San Giacomo and Wymer 1997).

CAIROX ZM®

Information on CAIROX ZM® Free-Flowing Grade Potassium Permanganate, a proprietary potassium permanganate compound that is also registered for control of zebra mussels, is provided as an example of the use of this chemical. CAIROX ZM is effective in the control of zebra mussels, biofilm, and other biofoulants such as plankton, algae, and microorganisms in raw water intake lines of drinking water and industrial water treatment systems.

Chemical Name and Formulations

a. *Chemical name*: Potassium permanganate, $KMnO_4$

b. *Formulation*: CAIROX ZM Free-Flowing Grade Potassium Permanganate

- Potassium permanganate 97 percent
- Granular
- Meets American Water Works Association Standard B 603 (1993).

c. *Source*: Carus Chemical Company, Carus Corporation
315 Fifth Street
P.O. Box 599
Peru, IL 61354-0599
(815) 223-1500

d. EPA Registration No.: 8429-9

Mode of Action

MnO_4^- is not the thermodynamically stable form of manganese in water; thus, permanganate tends to oxidize very slowly in water with the evolution of oxygen:

$$4\ MnO_4^- + 4\ H^+ \rightarrow 4\ MnO_2 + 2\ H_2O + O_2$$

Potassium permanganate has oxidizing activity. It has been observed that adult mussels retract their siphons while potassium permanganate is passing through water.

Application Strategies

Use this compound on-line in continuous dosage to eliminate mussels.

Add CAIROX ZM to raw water intake lines as early in the treatment system as possible.

It can be combined with chlorine.

Timing of Application

Apply this compound during summer mussel season, especially during the veliger settling phase.

Application Rates

Concentrations of CAIROX ZM required for oxidation of contaminants, typically up to 5 mg L^{-1}, are sufficient for the control of zebra mussels and other biofoulants.

Monitor to maintain a low residual concentration of less than 1 mg L^{-1} at the point of entry into a treatment plant.

Actual solubility in local water and effective doses should be determined using laboratory jar tests (Carus 1997).

Klerks, Fraleigh, and Stevenson (1993) showed that static exposure of 2.5 mg L^{-1} potassium permanganate resulted in 27 percent mortality of veligers, while in flow-through exposures veligers decreased by 90 percent with this concentration.

Balog et al. (1995) reported plans for the use of intermittent treatments at 0.35 mg L^{-1} residual level for at least 30 min in the treatment area for a municipal water treatment plant.

CAIROX ZM can be followed by chlorine.

Maximum Water Concentration

American National Standards Institute/National Sanitation Foundation Standard 60-1997 (1997) gives maximum use level as 50 mg L^{-1}.

Ensure that residues of manganese in finished potable water are limited to no more than 0.05 mg L^{-1}.

Use Restrictions

Ensure that residues of manganese in finished potable water are limited to no more than 0.05 mg L^{-1}.

A filtration plant residual of less than 0.25 mg L^{-1} (ppm) is needed to prevent pink discoloration of drinking water.

Do not use in facilities discharging directly or indirectly to estuarine or marine environments. Do not discharge effluent containing this product into lakes, streams, ponds, or public waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and the permitting authority has been notified in writing prior to discharge.

Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Timing of Results

Treatment effects on individuals occur within a few days. Continue treatment to system to maintain efficacy.

Toxicological Data

The Signal Word is Danger.

Toxicology of potassium permanganate to aquatic species is listed in the accompanying tabulation.

Precautions

Take the following precautions with this compound:

a. Strong oxidizer; keep from contact with combustible materials.

b. Corrosive; causes eye and skin damage. Avoid contact with eyes, skin, mucous membranes.

Aquatic Toxicity of Potassium Permanganate		
Organism	**Test/Water Condition**	**Level, mg L^{-1}**
Rainbow trout	96-hr LC_{50} 12 ºC 160-180 mg L^{-1} $CaCO_3$ pH 8.1	1.72
Channel catfish	96-hr LC_{50} 12 ºC 160-180 mg L^{-1} $CaCO_3$ pH 7.8	1.00
Rainbow trout	96-hr LC_{50} 12 ºC soft water pH 7.5	1.80
Channel catfish	96-hr LC_{50} 12 ºC soft water pH 7.5	0.75
Note: from Marking and Bills 1975.		

c. Prolonged inhalation of manganese compounds above the permissible exposure limit may cause lung irritation and central nervous system disorders.

d. When handling, use safety goggles, rubber gloves, and respirators. Avoid breathing dust.

e. Do not take internally; harmful if swallowed.

f. Toxic to birds and aquatic invertebrates.

Field Instructions and Guidance on Operational Applications

CAIROX is available in 25-kg pails, 50-kg kegs, 150-kg drums, 1,500-kg cycle-bins, and bulk up to 21,772 kg (48,000 lb).

Under normal conditions, CAIROX is stable. It will keep indefinitely if stored in a cool, dry area in a closed container. Avoid contact with acids, peroxides, and all combustible organic or readily oxidizable materials including metal powders. With hydrochloric acid, chlorine gas is liberated. It may decompose if exposed to heat. Store at less than 302 ºF (150 ºC).

Where exposure to airborne CAIROX may exist, a user should wear goggles, rubber or plastic gloves, and an approved National Institute for Occupational Safety and Health/Mine Safety and Health Administration dust and mist respirator. Normal clothing that covers arms and legs and a rubber or plastic apron are suitable attire. Always provide ventilation in the work area.

Following exposure to CAIROX potassium permanganate, brown stains of manganese dioxide often form on the skin as a natural decomposition product. These stains are harmless and can be removed using a solution composed of 3 parts 3 percent hydrogen peroxide, 4 parts 5 percent food grade white vinegar, and 3 parts tap water. Wash off excess when the stain is gone. Do not use if skin becomes red or irritated, or on sensitive tissue such as eyes, mucous membranes, open wounds, or burns.

Adjuvant/Detoxicant/Deactivant Use

None is specified.

Application Techniques

Dry flowable product can be poured from pails or handled in bulk.

Antidote Information

Use the following antidotes:

a. *If exposed to this product*: flood eyes with water for at least 15 minutes; wash skin thoroughly with soap and water; remove clothing for washing.

b. *If swallowed*: if the victim is conscious, give one or two glasses of water. Never give anything by mouth to an unconscious or convulsing person.

c. *If in eyes*: flood eyes with water for at least 15 minutes, holding the lids open. Do not attempt to use chemical antidotes.

d. *If on skin*: wash thoroughly with soap and water.

e. *If inhaled*: remove victim to fresh air.

f. *Emergency telephone*: 1-800-435-6856
CHEMTREC: 1-800-424-9300

References

American National Standards Institute/National Sanitation Foundation. (1997). "Drinking water treatment chemicals - health effects," ANSI/NSF 60-1997, Ann Arbor, MI.

American Water Works Association. (1993). "Potassium Permanganate," Document Number AWWA B603-93, Washington, DC.

Balog, G. G., Neimeyer, T. F., Davis, L. S., Sokhey, A. A., Scott, D. E., and Custodio, O. (1995). "Baltimore City adopts a proactive approach to zebra mussel control using potassium permanganate." *Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 14-21, 1995. The Professional Edge, Pembroke, Ontario, Canada, 9-21.

Carus Chemical Corporation. (1997). "CAIROX ZM® Free-Flowing Grade Potassium Permanganate: Label," Peru, IL, 2 pp.

Carus Chemical Corporation. (1999). "CAIROX ZM® Free-Flowing Grade Potassium Permanganate: MSDS," Peru, IL, 2 pp.

Claudi, R., and Mackie, G. L. (1994). *Practical manual for zebra mussel monitoring and control.* Lewis Publishers, Boca Raton, FL, 227 pp.

Fraleigh, P. C., Van Cott, W. R., Wenning, M. E., and DeKam, J. A. (1993). "Effects of hypochlorite, permanganate, chloride dioxide, and chloramine on zebra mussel settling." *Third International Zebra Mussel Conference, Agenda and Abstracts*, Toronto, Ontario. February 23-26, 1993.

Klerks, P. L., Fraleigh, P. C., and Stevenson, R. C. (1993). "Controlling zebra mussel (Dreissena polymorpha) veligers with three oxidizing chemicals: Chlorine, potassium permanganate, and peroxide + iron." *Zebra mussels: Biology, impacts, and control.* T. F. Nalepa and D. W. Schloesser, eds., Lewis Publishers, Boca Raton, FL, 621-642.

Marking, L. L., and Bills, T. D. (1975). "Toxicity of potassium permanganate to fish and its effectiveness for detoxifying antimycin," *Transactions of the American Fisheries Society* 104(3), 579-583.

San Giacomo, R., and Wymer, M. W. (1997). "Successful applications of zebra mussel treatment, excluding chlorine." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 501-506.

4 Nonoxidizing Molluscicides

This group of chemicals includes most of the nongeneric and commercial formulations that have been recently registered specifically for use in zebra mussel control. Their chemistry and activity differ from the oxidizing compounds, and they provide a different range of potential applications.

Quaternary and Polyquaternary Ammonium Compounds

These organic compounds comprise chemicals known as quaternary ammonium compounds (QAC's) and polyquaternary ammonium compounds (poly-QACs, or polyquats).

BULAB 6002

BULAB® 6002 is a liquid cationic polyquaternary ammonium compound, a straight-chain ionene polymer with positively charged nitrogen atoms in the backbone of its polymeric chain (McMahon, Shipman, and Long 1993). It is used for algae control in swimming pools and as a microbicide for the control of microorganisms in commercial and industrial water systems. It also is an effective molluscicide and can prevent biofouling by mollusks (McMahon and Lutey 1988; McMahon, Shipman, and Ollech 1989; Waller et al. 1993; Buckmann Laboratories, Inc., 1998). It is effective with or without the use of chlorine. It is approved for use in drinking water by the American National Standards Institute/National Sanitation Foundation (ANSI/NSF 1997).

Chemical Name and Commercial Formulations

This compound has the following characteristics:

a. Chemical name:

- poly[oxyethylene(dimethyliminio)ethylene(dimethyliminio)ethylene dichloride
- $C_{10}H_{24}N_2OC_{l2}$
- Also known as PQ1 or WSCP

b. Formulation: BULAB 6002

- 60 percent polymeric quaternary ammonium
- Water-soluble liquid; U.S. Environmental Protection Agency Reg. No. 1448-42

c. Source: Buckman Laboratories, Inc.
1256 McLean Boulevard
Memphis, TN 38108
(901) 278-0330
1-800-BUCKMAN

Mode of Action

As a poly-QAC, BULAB 6002 binds to negatively charged surfaces including those of microorganisms and mollusk membranes. It is not detected by mussels as a noxious compound and closure response is not provoked; kill can occur quickly.

Application Strategies

The compound can be applied as short-term, continuous, or intermittent treatments.

Timing of Application

The compound is generally used for one-time application, end of season, or periodic use.

Application Rates

To control mollusks in recirculating or once-through cooling water and industrial systems using continuous or intermittent application, add BULAB 6002 at dosage rates of 0.2 to 2.2 fluid ounces of BULAB 6002 per 1,000 gallons of water (15.6 ml to 172 ml to 10,000 L), or 2 to 20 ppm product. Addition should be made continuously or intermittently to the intake water. Continuous addition is required for noticeably fouled systems.

Intermittent feeding is used to maintain control.

Mollusk fouling has been prevented by concentrations as low as 2 ppm.

Initial concentrations of 2 to 5 mg L^{-1} can be used up to 21 days. The long-term limit is 0.5 mg L^{-1}.

The following tabulation summarizies laboratory studies showing efficacies of this product and the relationship of exposure time to concentration. Martin, Mackie, and Baker (1993a) showed that toxicity was temperature dependent.

Activity of BULAB 6002 on Zebra Mussel

Concentration mg L^{-1}	LT_{50}, hr		LT_{100}, hr		MTD, hr	
	MMB	MSL	MMB	MSL	MMB	MSL
1.0	168	499	250	680	514	175
2.0	148	216	250	313	231	166
4.0	108	174	196	244	189	123
8.0	96	124	144	197	147	107

Note: MMB = Martin. Mackie, and Baker (1993b).
MSL = McMahon, Shipman, and Long (1993).
LT_{50}, LT_{100} = time to percent mortality.
MTD = mean time to death.

Maximum Water Concentration

The long-term limit in potable water is 0.5 mg L^{-1} (ppm) maximum.

Use Restrictions

Initial concentrations of 2 to 5 mg L^{-1} can be used up to 21 days. The long-term limit is 0.5 mg L^{-1} in water.

This product is toxic to fish and aquatic organisms at certain concentrations. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance, contact the governing State Water Board or Regional Office of the U.S. Environmental Protection Agency.

It is approved for potable water systems for mollusk treatment at 0.5 ppm maximum (ANSI/NSF 1997).

Use in closed systems is unrestricted.

Timing and Appearance of Effects

Lethality is seen within 5 days, depending on concentration. Continue applications as recommended for full treatment effect.

Toxicological Data

The following tabulation lists the toxicity of BULAB 6002:

Toxicity of Active Product, BULAB 6002		
Organism	**Test, LC_{50}**	**Limit, mg L^{-1}**
Daphnia magna	48-hr	0.37
Bluegill sunfish	96-hr	0.21
Rainbow trout	96-hr	0.047
Fathead minnow	96-hr	0.26
Sheepshead minnow	96-hr	> 600
Mysid shrimp	96-hr	13.0
Quahog clam	96-hr	0.35
Note: From Buckman Laboratories, Inc., 1997.		

BULAB 6002 does not degrade readily in water but adsorbs strongly to sediments, clay particles, organic matter and other negatively charged surfaces, so that water column concentrations are normally very low: adsorption and biodegradation are probably the dominant fate processes in aquatic systems (Electric Power Research Institute 1993).

Precautions

This compound is harmful if swallowed. Avoid breathing vapors. Avoid contact with skin, eyes, or clothing.

It is a mild irritant to eyes, nonirritating to skin, and may cause irritation or corrosion of mucous membranes and lungs.

Rubber gloves, indirect ventilation goggles, body-protective clothing, and rubber safety shoes are required.

Field Instructions and Guidance on Operational Applications

See Buckman Laboratories, Inc. (1997), for extensive list of construction materials satisfactory for use in handling BULAB 6002.

Adjuvant Use

There is no adjuvant use. It can be applied with or without chlorine.

Application Techniques

Apply to intake water, at a point of maximum water agitation.

Antidote Information

Use the following antidotes:

a. *If swallowed*: do not induce vomiting. Rinse with copious amounts of water or milk. Irrigate the esophagus and dilute the stomach content by slowly giving one to two glasses of water or milk. If person is comatose or convulsing, do not give fluids by mouth. Get medical assistance immediately; take individual to nearest medical facility.

b. *If in eyes*: rinse eyes immediately for at least 15 minutes with copious amounts of water. Seek medical attention.

c. *If on skin*: rinse with large amounts of water and wash with soap and water. Remove contaminated clothing and wash before reuse. If irritation occurs, seek medical attention.

d. *If inhaled*: move person to a well-ventilated place and apply artificial respiration if required. Call a physician.

e. *Emergency phone number, 24 hour*: (901) 767-2722.

References

American National Standards Institute/National Sanitation Foundation. (1997). "Drinking water treatment chemicals - health effects," ANSI/NSF 60-1997, Ann Arbor, MI.

Buckman Laboratories, Inc. (1997). Bulab 6002: MSDS. Memphis, TN, 6 pp.

Buckman Laboratories, Inc. (1998). Bulab 6002: Label. Memphis, TN, 6 pp.

Electric Power Research Institute. (1993)."Hazard identification of commercially available biocides to control zebra mussels and Asiatic clams," TR-103175, Syracuse Research Corporation, Syracuse, NY.

Martin, I. D., Mackie, G. L., and Baker, M. A. (1993a). "Acute toxicity tests and pulsed-dose delayed mortality at 12 and 22 EC in the zebra mussel *Dreissena polymorpha*," *Archives of Environmental Contamination and Toxicology* 24, 389-398.

Martin, I. D., Mackie, G. L., and Baker, M. A. (1993b). "Control of the biofouling mollusc, *Dreissena polymorpha* (Bivalvia: Dreissenidae), with sodium hypochlorite and with polyquaternary ammonia and benzothiazole compounds," *Archives of Environmental Contamination and Toxicology* 24, 381-388.

McMahon, R. F., and Lutey, R. W. (1988). “Field and laboratory studies of the efficacy of poly[oxyethylene(dimethyliminio)ethylene(dimethyliminio)ethylene dichloride as a biocide against the Asian clam, *Corbicula fluminea*.” *Proceedings of the Service Water System Reliability Improvement Seminar*, Electric Power Research Institute, Palo Alto, CA, 11 pp.

McMahon, R. F., Shipman, B. N., and Long, D. P. (1993). “Laboratory efficacies of nonoxidizing molluscicides on the zebra mussel (*Dreissena polymorpha*) and the Asian clam (*Corbicula fluminea*).” *Zebra mussels: Biology, impacts and control*. T. F. Nalepa and D. W. Schloesser, ed., Lewis Publishers, Boca Raton, FL, 575-598.

McMahon, R. F., Shipman, B. N. and Ollech, J. A. (1989). Effects of two molluscicides on the freshwater bivalves, *Corbicula fluminea* and *Dreissena polymorpha*.” *Service Water System Reliability Improvement Seminar: Addendum*. Electric Power Research Institute, Palo Alto, CA, 55-81.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). “Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms,” *J. Great Lakes Res.* 19, 695-702.

Calgon H-130M

This compound is a nonoxidizing liquid organic compound containing a solution of polyquaternary alkyl ammonium registered for use as a molluscicide in industrial once-through freshwater cooling water systems. Calgon H-130M controls zebra mussel and the Asiatic clam in veliger, juvenile, and adult forms. Because of its need for proper deactivation prior to discharge, it is sold only as part of a complete Calgon mollusk treatment application service, and is to be used only with supervision from a Calgon representative.

Chemical Name and Commercial Formulations

This compound has the following characteristics:

a. *Chemical Name*: Didecyldimethylammonium chloride, known as DDMAC.

b. *Formulation*: H-130M; 50 percent DDMAC; liquid, U.S. Environmental Protection Agency Registration No. 6836-203-10445.

c. *Source*: Calgon Corporation
P.O. Box 1346
Pittsburgh, PA 15230-1346
(412) 777-8000
Health and Environmental Affairs: (412) 494-8000

Mode of Action

Antifouling activity results from coating, surfactant action.

Application Strategies

Slug feed for once-through systems.

Timing of Application

Apply up to four times per year as periodic treatment to limit accumulation of adults.

Application Rates

Apply 1 to 10 ml H-130M per 1,000 L (1-10 ppm) water (0.15 to 1.5 fluid ounces per 1,000 gal of water) to give 1 to 10 mg L^{-1} (ppm) (Calgon Corporation 1996a).

Treatment is typically a 24-hr feed period at 1.5 ppm to the inlet of the plant to maintain a residual at the discharge of 0.5 ppm. Actual treatment durations may

vary from site to site dependent on water temperature and other site-specific conditions.

Do not apply H-130M more than four times per year. The duration of the treatment must not exceed 120 hr per application.

Refer to the following tabulation for efficacy data.

Toxicity of Active Ingredient (DDMAC) to Target Organisms

Organism/Size	Median Lethal Concentration	
	48-hr Test mg L^{-1}	48-hr Post Exposure mg L^{-1}
Zebra mussel, 20- to 25-mm diameter	0.85	0.38
Zebra mussel, 5- to 8-mm diameter	1.12	0.59
Threehorn wartyback	6.12	3.72

Note: from Waller et al. 1993; Fisher et al. 1994.

Maximum Water Concentration

H-130M must be deactivated prior to discharge.

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, ocean or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit and notification of the permitting authority in writing prior to discharge.

Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Use Restrictions

H-130M is toxic to fish.

This compound requires proper deactivation prior to discharge.

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, ocean or other waters unless in accordance with the requirements of an NPDES permit and notification of the permitting authority in writing prior to discharge.

Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance

contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Do not apply H-130M more than four times per year. The duration of the treatment must not exceed 120 hr per application.

Use of this product in either public/municipal or single- or multiple-family private/residential potable/drinking water systems is strictly prohibited. Use of this product in any cooling water system that discharges effluent within 1/4 mile of either a public/municipal or single- or multiple-family private/residential potable/drinking water intake is strictly prohibited.

Do not use water containing residues from use of this product to irrigate crops used for food or feed.

Do not contaminate water, food, or feed by storage or disposal.

Timing and Appearance of Effects

Mussels are affected within 2 days of application (see preceding tabulation).

Toxicological Data

The Signal Word is Danger

The following tabulation describes the aquatic toxicity of this compound.

Toxicity of Active Ingredient (DDMAC) of H-130M

Organism	Test	Median Lethal Concentration of H-130M, mg L^{-1}
Rainbow trout	96-hr	1.1
Coho salmon	96-hr	1.0
Daphnia magna	48-hr	0.094
Mysid shrimp	96-hr	0.069
Bluegill sunfish	96-hr	0.32 - 0.59

Note: from Calgon Corporation 1996b

Precautions

This compound is corrosive and harmful or fatal if swallowed. Do not get in eyes or on skin or clothing. It can cause severe damage to eyes and skin. Wear safety glasses, goggles, or face shield and rubber gloves when handling. Avoid breathing spray mist.

H-130M is a flammable/combustible liquid and vapor. Do not use, pour, spill, or store near heat or open flame.

Do not use or compound H-130M with any reducing or oxidizing agents (such as calcium hypochlorite, solid perchlorate, or nitric acid) since such mixtures may be explosive. Do not use in conjunction with soap or any anionic wetting agent.

Field Instructions and Guidance on Operational Applications

Rate of adsorption onto target mollusks is temperature dependent.

H-130M residual is monitored throughout the plant system and prior to discharge using a test procedure sensitive down to 20 ppb. A composite water sample is sent to an outside laboratory, and test results are provided to the customer to verify that effluent water is safe.

Recommended handling materials for pump and piping are polypropylene, polyethylene, Hypalon, stainless steel, epoxy phenolic-lined steel, or isophthalic polyester resins.

Adjuvant/Detoxicant/Deactivant Use

The compound must be deactivated prior to discharge from the system using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product.

The proprietary product CA-35 is also used to deactivate H-130M in plant water prior to discharge to the environment. The feed rate of the CA-35 is typically 5 ppm for every 1 ppm of H-130M fed to the inlet water. CA-35 feed continues at least 2 hr past the time when the H-130M is discontinued, to assure complete deactivation of biocide remaining in the system.

Application Techniques

H-130M is fed as close to the raw water inlet as possible without risking the release of the chemical into the environment.

H-130M should be fed as received if the product is fed directly from drum or pail. If the product is slug-fed by hand, prepare a 2:1 mixture of water to biocide. Prepare mixture by slowly adding product to the mix water. This procedure minimizes foaming during dilution and when feeding the diluted product to water. Apply at a point in the system where the product will be uniformly mixed.

Antidote Information

In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes. In case of contact with eyes, call a physician immediately.

If the compound is swallowed, immediately drink 3 to 4 glasses of milk, or egg whites, or gelatin solution; if these are not available, drink a large quantity of water. Call a physician immediately.

Note to physician: probable mucosal damage may contraindicate use of gastric lavage. Measures against circulatory shock, respiratory depression, and convulsion may be needed.

References

Calgon Corporation. (1996a). "H-130M: Label." Pittsburgh, PA. 1 p.

Calgon Corporation. (1996b). "H-130M: Material Safety Data Sheet." Pittsburgh, PA. 7 pp.

Fisher, S. W., Dabrowska, H., Waller, D. L., Babcock-Jackson, L., and Zhang, X. (1994). "Sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to candidate molluscicides," *J. Shellfish Res.* 13: 373-377.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). "Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms," *J. Great Lakes Res.* 19:695-702.

Clam-Trol™

The primary active ingredients of these products are cationic surfactants of the n-alkyldimethyl-benzyl ammonium chloride (ADBAC) family. These quaternary ammonium compounds have various length carbon chains and are short-lived in plant systems and the environment because of rapid absorption onto anionic substrates and sediments in natural aquatic ecosystems. They are used for cooling and service water systems.

Chemical Name and Commercial Formulations

The active ingredients of these products have the following chemical names:

a. *ADBAC, Quat*: n-Alkyl (C12, C14, and C16) dimethylbenzyl ammonium chloride

b. *DGH*: Dodecylguanidine hydrochloride

Their formulations are as follows:

a. Clam-Trol CT-1:

- 8 percent n-alkyl (C12-40 percent, C14-50 percent, C16-10 percent) dimethylbenzyl ammonium chloride
- 5 percent dodecylguanidine hydrochloride
- Liquid
- U.S. Environmental Protection Agency Registration No. 3876-145

b. Clam-Trol CT-2:

- 50 percent n-alkyl (C12-50 percent, C14-40 percent, and C16-10 percent) dimethylbenzyl ammonium chloride
- Liquid

c. Clam-Trol CT-4

- 10 percent n-alkyl (C12-50 percent, C14-40 percent, and C16-10 percent) dimethylbenzyl ammonium chloride
- Liquid

d. *Source*: BetzDearborn, Inc.
4636 Somerton Road
Trevose, PA 19053
Information: (215) 355-3300
Emergency: 1-800-877-1940

Mode of Action

These products work by adsorbence to system components. Mussels do not detect them as noxious compounds and they do not close their shells. This allows them to be affected and killed quickly, with significant mortality in 4 to 24 hr.

Clam-Trol causes detachment of adults and is effective on mollusks at all life stages. It also controls microfouling organisms.

The formulations have the following effects:

a. *CT-1*: For control of mollusca, barnacles, hydrozoa, bryozoa, bacteria, fungi, and algae. Effective on zebra mussels, Asiatic clams, ribbed mussels, blue mussels, and most other freshwater and saltwater mollusks. Also controls bacterial, fungal, and algal slime.

b. *CT-2*: Effective on freshwater and saltwater mollusks, including zebra mussels, Asiatic clams, ribbed mussels, blue mussels, and oysters. Assists in controlling microbial growth including algae, bacteria, and fungi.

c. *CT-4*: Effective on zebra mussels and Asiatic clams.

Application Strategies

Apply these compounds as follows:

a. Continuous, intermittent, or as needed. Frequency of feeding and duration of treatment depend upon severity of problem.

b. Periodic, short-term (6 to 24 hr) applications to water on a proactive basis to prevent mollusks from growing to a fouling size.

c. Intermittent or slug method: when system is noticeably fouled, apply initial dose to achieve control. Repeat the process periodically to inhibit recolonization by larvae (veligers) and juvenile clams and mussels.

d. Applications may be able to be conducted off-line or at reduced flow.

e. Registered use areas in which to control mollusks with these products are as follows:

(1) CT-1: recirculating and once-through cooling systems, influent cooling systems, auxiliary water and wastewater systems, fire protection systems, intake pump bays and intake screen area, storage tanks and associated piping, settling ponds or lagoons, transport spillways or canals.

(2) CT-2: recirculating and once-through cooling systems, service water, auxiliary water, influent, fire protection, and wastewater systems. For auxiliary water/service water and wastewater systems,

may be added to the system water or by spraying onto a waste pile as needed.

(3) CT-3: cooling towers and once-through freshwater cooling systems.

(4) CT-4: once-through freshwater cooling systems.

Sensitivity of various life stages has been studied by Waller et al. (1993) and Fisher et al. (1994).

Timing of Application

They may be applied continuously, intermittently, or as needed during the season of activity.

Application Rates

Rates vary by product formulation and system. See accompanying tabulations (BetzDearborn, Inc., 1988, 1993a, 1993b). Badly fouled systems must be cleaned before treatment is begun.

Clam-Trol CT-1 is applied as follows:

a. Warmer water temperatures and longer contact times reduce the concentration of CT-1 needed for effective kills.

b. Product weight is 1 kg L^{-1} (8.5 lb/gal), and concentrations are based on product.

c. Apply as follows for recirculating cooling water systems:

(1) Intermittent or slug method: Initially when fouled, 360 g to 2.4 kg per 10,000 L water (0.3 to 2.0 lb per 1,000 gal of water), 36 to 240 ppm. Repeat until control is achieved. Subsequently, apply 180 g to 1.8 kg per 10,000 L water (0.15 to 1.5 lb per 1,000 gal of water), 18 to 180 ppm, every 3 days, or as needed to maintain control.

(2) Continuous feed method: Initially when fouled, 360 g to 2.4 kg per 10,000 L water (0.3 to 2.0 lb per 1,000 gal of water), 36 to 240 ppm. Subsequently, maintain 60 to 600 g per 10,000 L water (0.05 to 0.5 lb per 1,000 gal water), 6 to 60 ppm, in system.

d. Apply as follows for once-through industrial cooling water systems:

(1) Intermittent or slug method: Initially when fouled, 240 g to 1.2 kg per 10,000 L water (0.2 to 1.0 lb per 1,000 gal of water), 24 to 120 ppm, at minimum treatment intervals of 15 min. Repeat until control is achieved. Subsequently, 60 to 600 g per 10,000 L water

Application Methods for Clam-Trol Formulations

Product/System	Intermittent or Slug	Continuous Feed	Other
CT-1			
Recirculating cooling water	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.15 - 1.5 lb/1,000 gal 180 - 1.8 kg/10,000 L (18 - 180 ppm) Every 3 days/as needed	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm)	
Once-through industrial cooling water	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm) As needed	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.02 - 0.2 lb/1,000 gal 24 - 240 g/10,000 L (2.4 - 24 ppm)	
Auxiliary water/wastewater	Initial: 1.5 - 4 lb/1000 gal 1.8 - 4.8 kg/10,000 L for 4 to 8 hr 1 to 4 times/week (180 - 480 ppm) Subsequent: 0.75 - 2 lb/1000 gal 900 g - 2.4 kg/10,000 L (90 - 240 ppm)		Spray onto waste pile
CT-2			
Recirculating or once-through Industrial/commercial cooling water systems	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr		
Auxiliary water/service water and wastewater systems	0.3 to 1.3 lb/1,000 gal 360 g - 1.56 kg/10,000 L (36 - 156 ppm) for 4 - 8 hr 1 - 4 x/week Subsequent: 0.15 - 0.65 lb/1,000 gal 180 - 780 g/10,000 L 18 to 78 ppm		Spray onto waste pile
CT-4			
Once-through freshwater cooling systems	1.28 - 12.8 fl oz/1,000 gal 100 ml - 1 L/10,000 L 1 - 10 ppm ai Treat #120 hr # 4 times per year		

Application Rates of Clam-Trol Formulations for Various Water-Handling Systems

Water/System Application Method	Clam-Trol Product		
	CT-1	**CT-2**	**CT-4**
Recirculating Cooling Water			
Intermittent or slug	Initial: 0.3 - 2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.15 - 1.5 lb/1,000 gal 180 - 1.8 kg/10,000 L (18 - 180 ppm) Every 3 days/as needed	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr	
Continuous feed:	Initial: 0.3 -2.0 lb/1,000 gal 360 g - 2.4 kg/10,000 L (36 - 240 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm)		
Once-Through Freshwater Cooling			
Intermittent or slug	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.05 - 0.5 lb/1,000 gal 60 - 600 g/10,000 L (6 - 60 ppm) As needed	Initial: 0.016 - 0.166 lb/1,000 gal 20 - 200 g/10,000 L (2 - 20 ppm) Maintain 3 - 48 hr	1.28 - 12.8 fl oz/1,000 gal 100 ml - 1 L/10,000 L 1 - 10 ppm ai Treat #120 hr # 4 times per year
Continuous feed	Initial: 0.2 - 1.0 lb/1,000 gal 240 g - 1.2 kg/10,000 L (24 - 120 ppm) Subsequent: 0.02 - 0.2 lb/1,000 gal 24 - 240 g/10,000 L (2.4 - 24 ppm)		
Auxiliary Water/Service Water and Wastewater			
Intermittent or slug	Initial: 1.5 - 4 lb/1,000 gal 1.8 - 4.8 kg/10,000 L for 4 to 8 hr 1 to 4 times/week (180 - 480 ppm) Subsequent: 0.75 - 2 lb/1,000 gal 900 g - 2.4 kg/10,000 L (90 - 240 ppm)	Initial: 0.3 to 1.3 lb/1,000 gal 360 g - 1.56 kg/10,000 L (36 - 156 ppm) for 4 - 8 hr 1 - 4 x/week Subsequent: 0.15 - 0.65 lb/1,000 gal 180 - 780 g/10,000 L 18 to 78 ppm	
Other	Spray onto waste pile	Spray onto waste pile	

(0.05 to 0.5 lb per 1,000 gal of water), 6 to 60 ppm, as needed to maintain control.

(2) Continuous feed method: Initially when fouled, 240 to 1.2 kg per 10,000 L water (0.2 to 1.0 lb per 1,000 gal of water), 24 to 120 ppm. Continue until control is achieved. Subsequently, 24 to 240 g per 10,000 L water (0.02 to 0.2 lb per 1,000 gal of water), 2.4 to 24 ppm.

e. Apply as follows for auxiliary water and wastewater systems:

(1) Intermittent or slug method: 1.8 to 4.8 kg in 10,000 L water (1.5 to 4.0 lb per 1,000 gal of water), 180 to 480 ppm, in system water or in water being added to system, for 4 to 8 hr, 1 to 4 times per week or as needed to achieve control. Subsequently, 900 g to 2.4 kg in 10,000 L water (0.75 to 2.0 lb per 1,000 gal of water), 90 to 240 ppm.

(2) Can be sprayed onto a waste pile.

CT-2 is applied as follows:

a. Rates are given as weight of product, at 960 g L^{-1} (8.0 lb per gal). Concentrations are based on product.

b. Apply as follows for recirculating or once-through cooling water systems: add 20 to 200 g per 10,000 L water (0.016 to 0.166 lb per 1,000 gal of water), 2 to 20 ppm, based on water in the system or on flow rate through the system. Maintain this concentration for 3 to 48 hr.

c. Apply as follows for auxiliary water and wastewater systems:

(1) Intermittent or slug method: 360 - 1.56 kg/10,000 L (0.3 to 1.3 lb per 1,000 gal) of water in system or being added to system, 36 to 156 ppm, for 4 to 8 hr, 1 to 4 times per week or as needed to achieve control. Subsequently, use 180 to 780 g per 10,000 L (0.15 to 0.65 lb per 1,000 gal) of water, 18 to 78 ppm.

(2) Can be sprayed onto a waste pile.

CT-4 is applied as follows:

a. Rates are given as volume of product. Concentrations are based on active ingredient (ai) of quaternary compound (10 percent of product).

b. Apply as follows for once-through freshwater cooling water systems: 100 ml to 1 L per 10,000 L (1.28 to 12.8 fluid ounces per 1,000 gal) of water, 1 to 10 ppm at no more than 4 times per year and for no more than 120 hr per application.

Maximum Water Concentration

The maximum water concentration is designated in a National Pollutant Discharge Elimination System (NPDES) permit for individual facility. Notify the permitting authority in writing prior to discharge.

Notify the local sewage treatment plant authority before discharging effluent containing this product to sewer systems. Contact State Water Board or Regional Office of the U.S. Environmental Protection Agency (USEPA) for guidance.

Segment plantwide applications to reduce the amount of product appearing in effluent.

Use Restrictions

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, ocean or other waters unless in accordance with the requirements of an NPDES permit and notification of the permitting authority in writing prior to discharge.

Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance contact the State Water Board or Regional Office of the USEPA.

Effluent inactivation may be required to comply with State and Federal water quality criteria.

Do not use water containing residues from use of CT-2 and CT-4 to irrigate crops used for food or feed.

Use of CT-4 in public/municipal or single- or multiple-family private/residential potable/drinking water systems is strictly prohibited.

Use of CT-4 in any cooling water system that discharges effluent within 1/4 mile of either a public/municipal or single- or multiple-family private/residential potable/drinking water intake is strictly prohibited.

Do not contaminate water, food, or feed by storage or disposal.

Timing and Appearance of Effects

Treatment effects on individual mussels occur within a few days.

It is suggested that sites evaluate treatment effects by taking mussels from system and placing them into bioboxes for monitoring.

Toxicological Data

Sensitivity of nontarget organisms has been studied by Waller et al. (1993).

The Signal Word is DANGER.

The following tabulation lists the toxicology of Clam-Trol formulation:

Aquatic Toxicology of CLAM-TROL Formulations

Organism	CT-1		CT-2		CT-3	
	LC_{50} mg L^{-1}	NOEL mg L^{-1}	LC_{50} mg L^{-1}	NOEL mg L^{-1}	LC_{50} mg L^{-1}	NOEL mg L^{-1}
96-hr Flow-Through						
Rainbow trout	8.1	6.5	2	1.2	10	6
Fathead minnow	2.9	2.1	0.72	0.41	4	2
48-hr Flow-Through						
Daphnia magna	0.2	0.135	0.04	0.026	0.2	0.13
Ceriodaphnia	0.14	0.05	N/A		N/A	
96-hr Flow-Through						
Mysid shrimp	0.34	0.1	0.16	0.03	0.8	0.15
Sheepshead minnow	N/A		1.76	1	8.8	5
Menidia beryllina (Silversides)	N/A		0.62	0.35	3.1	1.75

Note: Data from BetzDearborn, Inc., 1998a, 1998b, 1998c.

Once adsorbed to bentonite clay, ADBAC is not harmful to aquatic organisms, benthic organisms, or microorganisms.

Precautions

This product can be toxic to fish and wildlife depending on dose.

It is corrosive. It causes eye and skin damage and is harmful if swallowed. Do not get into eyes, on skin, or on clothing. Do not inhale vapor or mist. Use with adequate ventilation.

Wear rubber gloves, goggles, or face shield when handling. Immediately remove and wash contaminated clothing before reuse. Wash thoroughly after handling.

Do not use, pour, or store near heat or open flame.

Field Instructions and Guidance on Operational Applications

Badly fouled systems must be cleaned before treatment is begun. Heavy infestations of mollusks should be physically removed by vacuuming, dredging, or scraping prior to Clam-Trol treatment.

A colorimetric field test is available to determine concentration of product in treated water.

Evaluate treatment effects by taking mussels from the system and placing them into bioboxes for monitoring.

CT-1 is compatible with stainless steel, copper alloys, and most common plastics and rubbers. Avoid the use of mild steel, low-density polyethylene, nitrile (Buna N), polyurethane, or Viton in handling the concentrated product.

CT-2 and CT-4 are compatible with all common engineering plastics and elastomers, except nylon. Preferred plastics are polyvinyl chloride, polythylene, and Teflon. Avoid linear high-density polyethylene for storage tanks. Preferred elastomers are butyl rubber, ethylene propylene rubber (ethylene propylene diene monomer) rubber, and natural rubber. Types 304 and 316 stainless steels are suitable for handling at temperatures below 120 ºF (49 ºC). Hastelloy or NMonel should be used instead of stainless steel for applications requiring prolonged exposure to undiluted product at temperatures > 120 ºF (49 ºC).

A listing of compatible feed equipment is available from BetzDearborn, Inc. (1990).

Adjuvant/Detoxicant/Deactivant Use

ADBACs undergo neutralization and detoxification by natural routes, but the process is accelerated by the addition of highly adsorbent, anionically charged materials.

An analytical test procedure to monitor product use and plant outfall levels is available from BetzDearborn, Inc. (1990).

For CT-1, the ammonium chloride quaternary ammonium compound and the DGH are readily neutralized by anionic materials such as clays (bentonite), silts, humic acids, suspended solids and cooling system surfaces. It can be actively detoxified by Betz® DT-1.

CT-2 and CT-4 can be inactivated using Betz DTS or DTG.

Do not discharge CT-4 without performing proper deactivation, using bentonite clay at 5 ppm or more of clay to 1 ppm of product.

Application Techniques

The products can be metered to a system for short application periods, using a suitably sized positive displacement pump.

Make additions of CT-2 to auxiliary water/service water and wastewater systems during the pumping operation and as close to the pump as possible to ensure adequate mixing.

CT-2 and CT-4 can also be metered into a flow of clean dilution water to facilitate use in a distribution header system.

If a closed-loop system can be set up to allow the molluscicide to be recirculated for the required period of time, the volume of chemical required can be significantly reduced.

Warmer water temperatures and longer contact times reduce the concentration of Clam-Trol required for effective treatment.

Recirculating or closed systems should be laid up for 12 to 24 hr after the system is treated. Where possible, blowdown from cooling towers should be suspended for 12 to 24 hr after the system is charged with an effective amount of product.

Segmenting plantwide applications reduces the amount of product that appears in plant effluent.

The product is available in 55-gal (108-L) drums, bulk, or semibulk containers.

Antidote Information

Use the following antidotes:

a. *Contact with skin*: wash immediately with plenty of soap and water. Immediately contact physician.

b. *Contact with eyes*: flush promptly and thoroughly with clear water for at least 15 minutes. Immediately contact physician.

c. *Ingestion*: immediately contact physician.

d. *Notice to physician*: mucosal damage may contraindicate the use of gastric lavage.

e. *Additional*: Measures against circulatory shock, respiratory depression, and convulsion may be needed.

f. *Emergency telephone*: 1-800-877-1940 (BetzDearborn).

References

BetzDearborn, Inc. (1988). “Label: Clam-Trol CT-1.” Trevose, PA. 2 pp.

BetzDearborn, Inc. (1990). “Betz CLAM-TROL® CT-1 molluscicide,” Product Facts PFP 083 9008, Trevose, PA.

BetzDearborn, Inc. (1993a). “Label: Clam-Trol CT-2.” Trevose, PA. 2 pp.

BetzDearborn, Inc. (1993b). “Label: Clam-Trol CT41.” Trevose, PA. 1 p.

BetzDearborn, Inc. (1998a). “Material Safety Data Sheet: CT-1.” Trevose, PA. 9 pp.

BetzDearborn, Inc. (1998b). “Material Safety Data Sheet: CT-2.” Trevose, PA. 8 pp.

BetzDearborn, Inc. (1998c). “Material Safety Data Sheet: CT-4.” Trevose, PA. 8 pp.

Fisher, S.W., Dabrowska, H., Waller, D. L., Babcock-Jackson, L., and Zhang, X. (1994). “Sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to candidate molluscicides,” *J. Shellfish Res.* 13: 373-377.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). “Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms,” *J. Great Lakes Res.* 19, 695-702.

MACROTROL 9210

MACROTROL 9210 and the more concentrated NALCO 9380 are water-soluble quaternary ammonium-based products of the n-alkyl dimethyl benzyl ammonium chloride type. They have penetrating and dispersing characteristics and act as nonoxidizing biocides. They are labeled for use in a wide range of water systems where they can control macroorganisms such as mollusks, clams, and barnacles, as well as microfoulants such as bacteria, fungi, and algae (Dobbs et al. 1995). They are effective in seawater as well as freshwater systems. Excess residues of NALCO 9380 and MACROTROL 9210 must be deactivated or detoxified prior to treated water discharge to a receiving stream.

Monitoring kits for use in checking zebra mussel veliger density and settling in a water system and experimental systems for determining adult mussel mortality under local water quality conditions are available from the source, Nalco Chemical Company (address given in next section).

Chemical Name and Commercial Formulations

These products have the following characteristics:

a. *Chemical name*: Ammonium chlorides

b. *Formulations*:

(1) MACROTROL™ 9210

- 5 percent alkyl (60 percent C14, 30 percent C16, 5 percent C12, 5 percent C-18) dimethyl benzyl ammonium chloride
- 5 percent alkyl (68 percent C12, 32 percent C14) dimethyl ethylbenzyl ammonium chlorides
- Liquid
- EPA Reg. No. 6836-57-1706

(2) NALCO® 9380

- 40 percent alkyl (60 percent C14, 30 percent C16, 5 percent C12, 5 percent C-18) dimethyl benzyl ammonium chloride
- 40 percent alkyl (68 percent C12, 32 percent C14) dimethyl ethylbenzyl ammonium chloride
- Liquid
- EPA Reg. No. 6836-234-1706

c. Source: Nalco Chemical Company
One Nalco Center
Naperville, IL 60563-1198
(630) 305-1000
Emergencies: 1-800-462-5378

Mode of Action

These products are corrosive to membranes, interfere with respiration, and are fast-acting.

Application Strategies

Use continuous or intermittent feed, depending on degree of system fouling and retention time. They can be applied off-line or on-line.

Use initial treatment to remove mussels from system, and follow with treatments as needed to maintain control.

The primary type of treatment program recommended for controlling zebra mussels with these products is eradication, to eliminate accumulated mussels.

Timing of Application

When system is noticeably fouled, apply to achieve control.

Monitor system to determine when to use subsequent treatments to maintain control.

For eradication, apply one to three times per year during warm season. Generally, apply immediately after the annual peak in reproduction (typically in June or July) and at the end of the season (October or November) (Nalco 1996a).

Application Rates

MACROTROL 9210. Note: Excess residual MACROTROL 9210 must be detoxified prior to discharge to a receiving stream by using the proprietary compound NALCO 1315 or by using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product (Nalco 1995a).

a. In recirculating, auxiliary cooling water, and wastewater systems:

(1) Initial dose: Add 0.08 to 0.8 lb per 1,000 gal water (10 to 100 ppm: 100 to 1,000 kg per 10,000 L). Repeat as necessary to achieve control.

(2) Subsequent dose: When control is evident, add 0.08 to 0.4 lb per 1,000 gal (10 to 50 ppm: 100 to 500 kg per 10,000 L), as needed to maintain control.

b. In once-through cooling water systems:

(1) Intermittent feed:

- *Initial dose:* When the system is noticeably fouled, add 0.08 to 0.8 lb per 1,000 gal water (10 to 100 ppm: 100 to 1000 kg per 10,000 L), based on system flow rates. The minimum treatment period should be 6 to 24 hr. Repeat as necessary to achieve control.
- *Subsequent dose:* When control is evident, add 0.04 to 0.4 lb per 1,000 gal (5 to 50 ppm: 50 to 500 kg per 10,000 L), based on system flow rates on an as-needed basis to maintain control. Frequency of feed should be tied to a monitoring program.

(2) Continuous feed:

- *Initial dose:* When the system is noticeably fouled, add 0.04 to 0.4 lb per 1,000 gal water (5 to 50 ppm: 50 to 500 kg per 10,000 L), based on system flow rates. Continue to feed until needed control is achieved.
- *Subsequent dose:* Maintenance control can be effective through continuous feed at 0.016 to 0.16 lb per 1,000 gal (2 to 20 ppm: 20 to 200 kg per 10,000 L), based on system flow rates.

NALCO 9380. Note: NALCO 9380 must be deactivated prior to discharge from the system by using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product or by using the proprietary compound NALCO 1315 (Nalco 1995b).

a. In recirculating, auxiliary cooling water, and wastewater systems:

(1) Initial dose: Add 0.2 to 1.7 fluid ounces per 1,000 gal water (1 to 12 ppm: 10 to 120 ml per 10,000 L). Repeat as necessary to achieve control.

(2) Subsequent dose: When control is evident, add 0.2 to 0.9 fluid ounces per 1,000 gal (1 to 6 ppm: 10 to 60 ml per 10,000 L), as needed to maintain control.

b. In once-through cooling water systems:

(1) Intermittent feed:

- *Initial dose:* When the system is noticeably fouled, add 0.2 to 1.7 fluid ounces per 1,000 gal water (1 to 12 ppm: 10 to 120 ml per 10,000 L) based on system flow rates. The minimum treatment period should be 6 to 24 hours. Repeat as necessary to achieve control.
- *Subsequent dose:* When control is evident, add 0.1 to 0.9 fluid ounce per 1,000 gal (0.6 to 6 ppm: 6 to 60 ml per 10,000 L),

based on system flow rates on an as-needed basis to maintain control. Frequency of feed should be tied to a monitoring program.

(2) Continuous feed:

- *Initial dose:* When the system is noticeably fouled, add 0.1 to 0.9 fluid ounce per 1,000 gal (0.6 to 6 ppm: 6 to 60 ml per 10,000 L), based on system flow rates. Continue to feed until needed control is achieved.
- *Subsequent dose:* Maintenance control can be effective through continuous feed at 0.03 to 0.3 fluid ounces per 1,000 gal (0.2 to 2.5 ppm: 2 to 25 ml per 10,000 L), based on system flow rates.

Maximum Water Concentration

Discharge concentrations of these products only in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit. Notify the permitting authority in writing prior to discharge. Notify the local sewage treatment plant authority before discharging effluent containing this product to sewer systems. For guidance, contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Excess residues of NALCO 9380 and MACROTROL 9210 must be detoxified prior to discharge.

Use Restrictions

Do not apply to potable or domestic water systems. Use in public or private potable water systems is strictly prohibited. Use in any cooling water system that discharges effluent within 0.25 mile of either a public or private potable water intake is prohibited.

Do not use water containing residues from use of this product to irrigate crops used for food or feed.

This product is toxic to fish and aquatic organisms at certain concentrations. Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and notification of the permitting authority in writing prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance, contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Excess residues of NALCO 9380 and MACROTROL 9210 must be detoxified prior to discharge. Deactivation is conducted by using bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product, or the product NALCO 1315.

NALCO 9380 cannot be sold or used in the State of California.

Timing and Appearance of Effects

Effects on respiratory activity of adult zebra mussels are seen within 24 hr (Nalco 1996a).

Toxicological Data

The Signal Word is Danger.

Toxicity is shown in the following tabulations:

Aquatic Toxicity of MACROTROL 9210 Using the 96-hr Static Acute Test

Organism	LC_{50}, mg L^{-1}
Mysid shrimp	0.9
Bluegill sunfish	5.2
Rainbow trout	9.3

From Nalco (1998).

Aquatic Toxicity of NALCO 9380 Using the 96-hr Static Acute Test

Organism	LC_{50}, mg L^{-1}
Mysid shrimp	0.115
Bluegill sunfish	0.644
Rainbow trout	1.162

From Nalco (1996b).

Precautions

This product is corrosive and harmful or fatal if swallowed.

It causes eye damage and skin irritation. Do not get in eyes, on skin, or on clothing. Wear goggles or face shield and rubber gloves when handling. Remove and wash contaminated clothing before reuse.

Field Instructions and Guidance on Operational Applications

After this product is used, it must be deactivated prior to discharge from the system using bentonite clay or the proprietary compound NALCO 1315 at a minimum ratio of 5 ppm clay to 1 ppm product.

Do not store the container on its side. Avoid creasing or impacting sidewalls of container.

Storage tanks should be constructed of polyvinyl chloride (PVC), carbon steel, or containers lined with Plasite 6000 or Plasite 7122. Feed lines and pumps should be constructed of PVC, Hypalong, Viton, Teflon, Buna-N, polypropylene, plexiglass, polyurethane, carbon steel, 304 stainless steel, or 316 stainless steel.

Adjuvant/Detoxicant/Deactivant Use

Excess residual MACROTROL 9210 and NALCO 9380 not deactivated by natural solids or turbidity in water must be detoxified prior to discharge to a receiving stream using the proprietary compound NALCO 1315 (a stabilized clay slurry) or bentonite clay at a minimum ratio of 5 ppm clay to 1 ppm product.

Feed clay continuously to the outfall to ensure maximum distribution and mixing with the water to be detoxified.

Application Techniques

Apply with a metering pump.

Apply at feed point as far upstream as possible to allow exposure to maximum number of mussels.

Antidote Information

Use the following antidotes:

a. *Eyes*: immediately flush with plenty of water for at least 15 minutes. Call a physician.

b. *Skin*: flush with plenty of water for at least 15 minutes.

c. *If swallowed*: drink a large quantity of milk, egg whites, or gelatin solution; if these are not available, drink large quantities of water. Avoid alcohol. Call a physician immediately.

d. *Note to physician*: probable mucosal damage may contraindicate the use of gastric lavage.

References

Dobbs, M. G., Cherry, D. S., Scott, J. C., and Petrille, J. C. (1995). "Environmental assessment of an alkyl dimethyl benzyl ammonium chloride (ADBAC) based molluscicide using laboratory tests." *Proceedings, Fifth International Zebra Mussel and Other Aquatic Nuisance Organisms Conference*, Toronto, Canada, February 14-21, 1995. The Professional Edge, Pembroke, Ontario, Canada, 87-101.

Nalco. (1995a). "MACROTROL 9210: Label," Nalco Chemical Company, Chicago, IL, 1 p.

Nalco. (1995b). "Nalco 9380: Label," Nalco Chemical Company, Chicago, IL, 1 p.

Nalco. (1996a). "Control of zebra mussels: Program profile," Nalco Chemical Company, Naperville, IL, 5 pp.

Nalco. (1996b). "Material safety data sheet: NALCO 9380," Nalco Chemical Company, Chicago, IL, 9 pp.

Nalco. (1998). "Material safety data sheet: NALCO 9210," Nalco Chemical Company, Chicago, IL, 9 pp.

VeliGON

High-charge cationic coagulant dimethyl diallyl ammonium chloride (DMDAAC) compounds have been used in water treatment plants as flocculators and clarification aids (Blanck, Mead, and Adams 1996), and have become the first molluscicide approved by the U.S. Environmental Protection Agency for use in domestic potable water systems. The various VeliGON formulations of these compounds differ in their molecular weight and cationic charge density. Floc allows settling out of veligers, and affects adults (Waller et al. 1993). The use of this compound in water treatment plants at concentrations for zebra mussel and *Corbicula* veliger control has been shown to reduce or eliminate the amount of alum coagulant required at the rapid mix area. This aids liquid/solid separation operations, resulting in less residual solids and soluble aluminum (sludge) in the plant effluent, and higher pH of finished water. The reduction in alum usage can improve the stability index and allow longer filter runs. Individual users must obtain specific discharge permits.

Chemical Name and Commercial Formulations

This compound has the following characteristics:

a. *Active ingredient*: poly (dimethyl diallyl ammonium chloride)

b. *Synonyms*: DMDAAC, pDADMAC, DDDMAC, DMDACC

c. *Formulations*:

(1) VeliGON™ CL-M

- 39.8 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-115

(2) VeliGON™ DL-M

- 17.5 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-121

(3) VeliGON™ L-M

- 19.8 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-116

(4) VeliGON™ LS-M

- 10 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-117

(5) VeliGON™ TL-M

- 19.8 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-118

(6) VeliGON™ T-2-M

- 33 percent poly (dimethyl diallyl ammonium chloride); liquid
- EPA Registration No. 10445-122

d. Source: Calgon Corporation
P.O. Box 1346
Pittsburgh, PA 15230-1346
(412) 777-8000
Health and Environmental Affairs: (412) 494-8000
Emergency: (412) 494-8000
For information, jim.farmerie@ecc.com.

Mode of Action

These quaternary ammonium compounds are aqueous solutions of medium [to low] molecular weight high-charge cationic polymers. They are flocculants and coagulants and produce a dense floc that works to prevent settling of zebra mussel veligers. They also affect adults, and it is suggested that these do not attach as firmly under treatment.

Application Strategies

Continuously feed during the spawning season at the intake of the raw water source.

The VeliGON compounds are approved for use in potable water treatment plant systems but can be applied only in a system where there is a treatment plant that includes a filter.

Timing of Application

Apply during the breeding season, when veligers are present in intake water due to mussel spawning activities. This varies by location. In the northeastern United States, spawning begins in the spring and continues through the summer.

Onsite monitoring is usually required to determine the start and duration of the spawning period in an area in order to optimize treatment timing.

Application Rates

Apply at a rate of 1 to 5 ppm on a continuous basis during the spawning/breeding season.

The various VeliGON compounds differ in molecular weight and cationic charge density. Choice of the most suitable product and determination of the application rate required for local water treatment systems is usually based on local veliger monitoring and optimal clarification effect in an on-site bench-scale test (jar test) under local water and site conditions.

DMDAAC has been shown to have a median lethal concentration LC_{50} at 96 hr for adult zebra mussels at between 1.5 and 3.0 mg L^{-1} (ppm) (Blanck, Mead, and Adams 1996).

The following tabulation lists the treatment rates for VeliGON formulatons:

Treatment Rates for VeliGON Formulations[1]

Product	Treatment to Intake Water	Concentration of Active Ingredient, ppm
VeliGON CL-M	0.3 to 3.3 fluid ounces per 1,000 gal 9 to 98 ml per 378,500 L	1 to 10
VeliGON DL-M	0.68 to 6.8 fluid ounces per 1,000 gal 20 to 200 ml per 378,500 L	1 to 10
VeliGON L-M	0.6 to 6.6 fluid ounces per 1,000 gal 18 to 195 ml per 378,500 L	1 to 10
VeliGON LS-M	1.2 to 13.2 fluid ounces per 1,000 gal 35.5 to 390 ml per 378,500 L	0.1 to 1
VeliGON TL-M	0.6 to 6.6 fluid ounces per 1,000 gal 18 to 195 ml per 378,500 L	1 to 10
VeliGON T-2-M	0.36 to 3.6 fluid ounces per 1,000 gal 11 to 106 ml per 378,500 L	1 to 10

[1]From product labels (Calgon Corporation 1995a, b, c, d, 1996a, b)

Maximum Water Concentration

Apply VeliGON products only in systems with a treatment plant that includes a filter.

Discharge into lakes, streams, ponds, or public waters only in accordance with the requirements of a National Pollutant Discharge Elimination System permit. Notify the permitting authority in writing prior to discharge.

Limit residues of poly (N,N-dimethyl diallyl ammonium chloride) in finished potable water to no more than 50 ppm (50 mg L^{-1}).

The VeliGON products are certified by the National Sanitation Foundation International to American National Standards Institute/National Sanitation Foundation (ANSI/NSF) Standard 60-1997 (ANS/NSF 1997) for use in potable

water as coagulation and flocculation drinking water treatment chemicals to these maximum doses/feed rates:

a. VeliGON CL-M: 25 mg L^{-1}

b. VeliGON DL-M: 57 mg L^{-1}

c. VeliGON L-M: 50 mg L^{-1}

d. VeliGON LS-M: 100 mg L^{-1}

e. VeliGON TL-M: 50 mg L^{-1}

f. VeliGON T-2-M: 23.8 mg L^{-1}

pDADMAC is certified to ANSI/NSF 60-1997.

Use Restrictions

Apply VeliGON products only in systems with a treatment plant that includes a filter.

This pesticide is toxic to fish and aquatic invertebrates. Do not use in facilities discharging directly or indirectly to estuarine or marine environments.

Do not discharge into lakes, streams, ponds, or public waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and notification of the permitting authority in writing prior to discharge.

Do not discharge effluent containing this product into sewage systems without previously notifying the local sewage treatment plant authority. For guidance contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Residues of poly (N,N-dimethyl diallyl ammonium chloride) in finished potable water should be no more than 50 ppm (50 mg L^{-1}).

Do not contaminate water, food, or feed by storage or disposal.

Timing and Appearance of Effects

Significant treatment effects on individuals are seen within 3 days.

Toxicological Data

Note: LC_{50} determinations without added suspended solids overestimate the true toxicity of cationic polymers. Suspended solids and other dissolved organic materials like humic acid are present in many natural waters, and reduce the effective concentration of the polymer and thereby its toxicity. A reduction in

toxicity is observed as shown in the following tabulation under turbid conditions (Calgon 1995e-h, 1996c,d).

Aquatic Toxicity Data for a 40 percent Solution of Poly(dimethyl diallyl ammonium chloride)

Organism	Test	Toxicity, mg L^{-1} (ppm)
Bluegill sunfish	96 hr LC_{50}	0.82 - 1.3
Rainbow trout	96 hr LC_{50}	0.37
Daphnia magna	48 hr LC_{50}	0.99 (in clear water)
		1.2 - 2.5 (in 50-ppm clay suspension)
		24.8 (in 1,000-ppm clay suspension)

Precautions

These products are not expected to cause eye or skin irritation, or to be toxic if swallowed. However, avoid contact with skin, eyes, or clothing. In case of contact, immediately flush eyes or skin with plenty of water. Get medical attention if irritation persists.

Use goggles; otherwise, no special personal protective equipment is required.

Field Instructions and Guidance on Operational Applications

Full-scale applications of VeliGON may allow greater reduction of primary coagulant than indicated in short, bench-scale preliminary tests due to longer time in the system.

Protect products from low temperatures. Maintain at 38 ºF (3 ºC) or higher. Store in heated buildings or heat-traced tanks to prevent freezing. Although products are freeze-thaw stable, stratification may occur upon freezing; they will become homogeneous again upon agitation.

The product is noncorrosive at use concentrations, but the undiluted product is moderately corrosive to iron and copper, including their alloys. Avoid exposing to carbon steel or copper. Do not store in stainless steel.

Storage tanks, chemical feed systems, and piping should be of high-density (HDPE) or cross-linked (XLPE) polyethylene, fiberglass (FRP) with polyester or vinylester resins, epoxy, or vinylester-lined steel. Pump liquid ends and piping should be constructed of polyethylene, propylene, polyvinyl chloride, chlorinated polyvinyl chloride, Kynar, 316 stainless steel, Viton, or Hypalon. Refer to manufacturer's recommendations.

Adjuvant/Detoxicant/Deactivant Use

None required.

Application Techniques

VeliGON may be metered into the flow and may be fed undiluted as long as in-line dilution is provided. Dilution to 1 percent as product is recommended to assure better contact of the coagulant with impurities in the water. Feed systems for undiluted material should be capable of handling 5,000-cps viscosity material. A Calgon SD, P-18, or MDS feed system is recommended by the manufacturer.

Addition of products should occur at a point sufficiently inside the intake pipe to prevent any release of VeliGON into the intake source (5 to 10 ft or 1.5 to 3 m for normal flow operations).

Addition should be made through a supply line placed inside the intake pipe or in some locations that will ensure that no contamination of public water occurs in the event of a break.

Feed exits must be equipped with a pressure check valve at the feed line exit to seal the feed line when intake flow stops.

Feed pumps must be designed to shut down when intake pumps stop. Planned shutdowns shall require feed pumps to be stopped 1/2 hr prior to shutdown.

Antidote Information

Use the following antidotes:

a. *If swallowed*: do not induce vomiting. Drink large quantities of water.

b. *If in eyes*: flood eyes with water for at least 15 minutes.

c. *If on skin*: wash thoroughly with soap and water.

d. *24-hour emergency telephone*: (412) 494-8000 (Calgon Corporation).

References

American National Standards Institute/National Sanitation Foundation. (1997). "Drinking water treatment chemicals - health effects," ANSI/NSF 60-1997, Ann Arbor, MI.

Blanck, C. A., Mead, D. F., and Adams, D. J. (1996). "Effective control of zebra mussels using a high molecular weight polymer." Abstract from the *Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 1996.

Calgon Corporation. (1995a). "Label: VeliGON CL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995b). "Label: VeliGON L-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995c). "Label: VeliGON LS-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995d). "Label: VeliGON TL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1995e). "Material Safety Data Sheet: VeliGON CL-M," Pittsburgh, PA. 6 pp.

Calgon Corporation. (1995f). "Material Safety Data Sheet: VeliGON L-M," Pittsburgh, PA. 6 pp.

Calgon Corporation. (1995g). "Material Safety Data Sheet: VeliGON LS-M," Pittsburgh, PA. 6 pp.

Calgon Corporation. (1995h). "Material Safety Data Sheet: VeliGON TL-M," Pittsburgh, PA. 6 pp.

Calgon Corporation. (1996a). "Label: VeliGON DL-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1996b). "Label: VeliGON T-2-M," Pittsburgh, PA. 1 p.

Calgon Corporation. (1996c). "Material Safety Data Sheet: VeliGON DL-M," Pittsburgh, PA. 6 pp.

Calgon Corporation. (1996d). "Material Safety Data Sheet: VeliGON T-2-M," Pittsburgh, PA. 6 pp.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). "Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms," *J. Great Lakes Res.* 19, 695-702.

Aromatic Hydrocarbons

These compounds are ring-structure organics with film-forming and surfactant activity and include formulations that have been used as biocides in industrial water-handling systems for many years.

BULAB 6009

This aromatic hydrocarbon product is used to control algae, bacteria, and fungi in industrial recirculating cooling water systems and to protect wood against dry or wet rot in cooling water towers. It is also used to prevent microorganism fouling in the petroleum industry.

Chemical Name and Formulations

This product has the following characteristics:

a. *Chemical name*: 2-(Thiocyanomethylthio)benzothiazole; known as TCMTB.

b. *Formulation*:

- BULAB® 6009
- 30 percent TCMTB; dispersable in water

c. *EPA Reg. No.*: 1448-55

d. *Source*: Buckman Laboratories, Inc.
1256 McLean Blvd.
Memphis, TN 38108
(901) 278-0330
1-800-BUCKMAN

Mode of Action

BULAB 6009 is corrosive to membranes. While some avoidance of the compound via shell closure has been seen, the majority of zebra mussels continue siphoning in the presence of TCMTB (McMahon, Shipman, and Long 1993).

Application Strategies

Treat waters of whole system periodically.

Timing of Application

Maintain concentrations during season of activity.

Application Rates and Duration

Use of 1 to 6 mg L^{-1} BULAB 6009 for once-through systems is the rate recommended for control of Asiatic clam, *Corbicula fluminea* (Electric Power Research Institute 1993).

The following tabulation summarizes laboratory studies showing efficacies of this product and the relationship of exposure time to concentration.

Activity of BULAB 6009 on Zebra Mussel

Concentration mg L^{-1}	LT_{50}, hr		LT_{100}, hr		MTD, hr	
	MMB	MSL	MMB	MSL	MMB	MSL
0.5	92	652	192	758	108	659
1.0	74	336	144	485	89	335
2.0	70	221	144	313	91	228
4.0	78	184	110	260	85	189

Note: MNB = Martin, Mackie, and Baker (1993)
MSL = McMahon, Shipman, and Long (1993)
LT_{50}, LT_{100} = time to percent mortality
MTD = mean time to death

Maximum Water Concentration

Discharge effluent containing this product only in accordance with the requirements of a National Pollutant Discharge Elimination System permit. Notify the permitting authority in writing prior to discharge.

Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance, contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Except for treatment purposes, do not apply directly to water, or to areas where surface water is present or to intertidal areas below the mean high-water mark.

Use Restrictions

This pesticide is toxic to fish.

Do not use in offshore or estuarine operations. In terrestrial uses, do not apply directly to open water, to areas where surface water is present, or to intertidal areas below the mean high-water mark.

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and written notification of the permitting authority prior to discharge. Do not discharge effluent containing this product to sewer systems without previously notifying the local sewage treatment plant authority. For guidance, contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Timing of Effects

Treatment effects are seen in 4 to 10 days (see previous tabulation).

Toxicological Data

BULAB 6009 is highly toxic to fish (Waller et al. 1993) (as shown in the following tabulation).

The Signal Word is Danger.

Toxicity of BULAB 6009

Organism	Test	Acute Aquatic Median Lethal Concentration, mg L^{-1}
Daphnia magna	48 hr	0.07
Fathead minnow	96 hr	0.037
Rainbow trout	96 hr	0.117

Precautions

Do not use or store near heat or open flame. Do not expose to extreme temperatures.

This product is corrosive to eyes, skin, and mucous membranes. Do not get in eyes, on skin, or on clothing. It causes irreversible eye damage. It also causes skin irritation, and may cause allergic skin reactions. It is harmful if swallowed.

Wear goggles or face shield and rubber gloves when handling. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash before reuse.

Field Instructions and Guidance on Operational Applications

Do not stack containers more than five drums high.

Do not heat or store above 122 ºF (50 ºC).

Drums should be opened in well-ventilated areas. Overpack leaking or damaged drums.

Spills should be absorbed in sawdust or sand and disposed of in a sanitary landfill. Keep container closed when not in use.

Adjuvant/Detoxicant/Deactivant Use

None required.

Application Techniques

Feed product at a point of strong agitation to ensure mixing.

Antidote Information

Use the following antidotes:

a. *If in eyes*: flush with plenty of water. Get medical attention.

b. *If on skin*: wash with plenty of soap and water. Get medical attention.

c. *If swallowed*: promptly drink a large quantity of milk, egg whites, or gelatin solution; if these are not available, drink large quantities of water. Avoid alcohol. Get medical attention.

d. *Note to physician*: no specific antidote is known. Probable mucosal damage may contraindicate gastric lavage. Treat symptoms.

e. *24-hour emergency number*: 901/767-2722.

References

Electric Power Research Institute. (1993). "Hazard identification of commercially available biocides to control zebra mussels and Asiatic clams," TR-103175, Syracuse Research Corporation, Syracuse, NY.

Martin, I. D., Mackie, G. L., and Baker, M. A. (1993). "Control of the biofouling mollusc, *Dreissena polymorpha* (Bivalvia: Dreissenidae), with sodium hypochlorite and with polyquaternary ammonia and benzothiazole compounds," *Archives of Environmental Contamination and Toxicology* 24, 381-388.

McMahon, R. F., Shipman, B. N., and Long, D. P. (1993). *Zebra mussels: Biology, impacts and control*. T. F. Nalepa and D. W. Schloesser, ed., Lewis Publishers, Boca Raton, FL, 575-598.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). "Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms," *J. Great Lakes Res.* 19, 695-702.

MEXEL 432

This mixture of aliphatic amine surfactants is an anti-fouling material that acts as a corrosion inhibitor and scale dispersant as well as having activity against freshwater and saltwater mussels and barnacles (Giamberini, Czembor, and Pihan 1994; Krueser, Vanlaer, and Damour 1997). It adsorbs to exposed surfaces and forms a protective anti-fouling film on internal components when present in circulating water. Once the material is adsorbed to a surface, it remains in place until it degrades, and this minimizes its presence in outfall.

Chemical Name and Formulations

This product has the following characteristics:

a. *Chemical name*: (Alkylamino)-3 aminopropane

b. *Formulation*:

- MEXEL® 432
- 1.7 percent (Alkyl amino)-3 aminopropane active ingredient (alkyl as in fatty acids of coconut oil); liquid
- 8.08 lb per gal; 970 g per L

c. *U.S. Distributor*: RTK Technologies, Inc.
P.O. Box 86622
Baton Rouge, LA 70879-6622
(225) 755-2194
RTKT1@aol.com
http://www.mexel.fr/mexel432.htm
EPA Registration No. 69100-1

Mode of Action

This hydrocarbon compound is a mixture of aliphatic hydrocarbons, with alcohol and amine functionality, in an aqueous emulsion. The amines act as surfactants, or “filming amines,” and adhere to wetted metal, plastic, concrete, and glass surfaces to form a film through which biofouling organisms cannot form an attachment. This preventive activity deters mussel infestation by repelling veliger settlement and adhesion to clean surfaces. The product gradually kills zebra mussels already in place by retarding byssal thread formation, adhering to and damaging gill surface membranes, and dispersing mussels. Thus, it prevents new infestations and gradually disperses existing infestations, and is used primarily to prevent infestations in a previously cleaned system.

Efficacy is due to presence on system surfaces, not in bulk water flow.

Application Strategies

This product is used for control of mollusks, including zebra mussels and clams, in nonpotable industrial water systems.

It is effective as an acute toxicant for systems that do not have continuous water flow (i.e., fire protection systems, standby facilities), but the preferred use is in closed delivery systems with daily dosage at sublethal levels.

Sites of application do not include freshwater cooling tower systems.

It may be applied to maintain clean systems or to treat systems that are already fouled. The system to be treated should first be cleaned of adult zebra mussels and then treated.

Treatment is usually on an intermittent basis, with normal frequency being once per day, or as needed to maintain control. Intermittent injection of low concentrations has been shown to have the potential for reducing molluscicide quantities while maintaining effectiveness (Giamberini, Czembor, and Pihan 1994).

Timing of Application

Initial application early in the season prior to veliger settlement is most effective, with continuation of daily dosing throughout warm weather.

Application Rate

Note: Dosage is a function of surface area rather than of water flow. See product label (Mexel S.A 1997b).

Standard dosage is for a short period each day, typically 4 ppm for 20 min per day.

Daily dose is determined by the amount of internal surface area to be protected and is calculated at approximately 0.033 lb or 15 g per day (1 lb or 454 g per month) per 100 ft^2 (9.3 m^2) of surface area. Inject dose into circulating water at a rate to achieve a concentration of 1 to 4 ppm: 1 to 4 pints of product for each 125,000 gal of water, or 1 to 4 L per 1,000,000 L water.

A dose of 12 mg L^{-1} per day in flow-through inhibits veliger settling.

Maximum Water Concentration

Do not discharge into environment or public waters.

Dosage in an operating system may be optimized to eliminate detectable concentrations in the effluent.

Use Restrictions

This pesticide is toxic to fish. Do not discharge into lakes, streams, ponds, or public waters unless in accordance with a National Pollutant Discharge Elimination System Permit. Do not flush to sewers. For guidance, contact the Regional Office of the U.S. Environmental Protection Agency.

Do not contaminate water, food, or feed by storage or disposal.

Timing of Effects

Treatment shows effects within a few days, but continuing treatment is required. Monitor treatment efficacy with bioboxes located at critical points in the system.

Toxicological Data

The Signal Word is Danger.

Note: Intermittent dosing at sublethal concentrations means that only organisms that remain within the treated system are at risk. The following tabulations list the aquatic toxicology for static and acute tests, respectively.

Aquatic Toxicology in Static Renewal Tests of MEXEL 432 Using Lake Superior Water Amended with 4.5 mg L^{-1} Humic Acids (mg L^{-1} = ppm)

Organism	Test	Median Lethal Concentration, mg L^{-1}
Rainbow trout	96-hr	11.0
Daphnia magna	48-hr	3.4
Fathead minnow	96-hr	8.06
Note: Data from Mexel S.A 1997a		

Aquatic Toxicology of MEXEL 432 in Acute Tests of Short Daily Exposures

Organism	Daily Exposure, min	Median Lethal Concentration, mg L^{-1}
Daphnia magna	5	26.9
	20	7.2
	80	3.0
Fathead minnow	5	13.1
	20	6.2
	80	2.8
Note: Data from Mexel S.A 1997a		

Precautions

MEXEL 432 is corrosive to skin, eyes, etc. It causes serious burns and is harmful if ingested.

Wear protective clothing, impermeable gloves, safety glasses plus goggles, or a face shield when handling undiluted product. Wash contaminated clothing thoroughly.

Field Instructions and Guidance on Operational Applications

Monitor treatment efficacy with bioboxes located at critical points in the system.

Do not store in low-density polyethylene, polypropylene, or copper, zinc, aluminum, and their alloys.

Store on impermeable surfaces within retention basin.

Avoid any discharge onto the ground. Protect sewers from possible discharges.

Monitor the presence of MEXEL 432 with colorimetric tests of grab samples, or with an electrode (Corroprobe®) measuring free corrosion potential as electrical potential.

Required dosage varies with the solids content of the water and with the temperature. When seasonal water turbidity is high, dosage may need to be increased; dosage may be reduced when water is cleaner. Colder water may require decreased dosage due to slower biodegradation rates. Biodegradation increases with increasing temperature and oxygen availability, and can be accelerated by agitation and by aeration.

Adjuvant or Detoxicant Use

No detoxification is required.

Application Techniques

Standard practice is to dose a system once a day, introducing MEXEL 432 into the water inlet with a metering pump for as long as it takes to inject the daily dosage, i.e., to produce a concentration of 1 to 4 ppm in the system. Under these conditions the product will not be present in the effluent in detectable concentrations.

Use a metering pump near a water inlet to pump the product into a small line that extends down to the inlet area of the main waterline, where it is dispersed into the inlet water stream. The metering pump may be interlocked with the main water pumps to eliminate the possibility of the product entering the environment in the event of reduced water flow. Inject product as near as possible to the inlet in order to protect as much of the inlet piping as possible.

Antidote Information

Use the following antidotes:

a. *Eyes*: hold eyelids open and flush with water. Wash with 0.5 percent acetic acid solution, and then rinse with water for 15 minutes. Consult an ophthalmologist.

b. *Skin*: wash with 2 percent acetic acid solution, then with plenty of soap and water. Get medical attention.

c. *If swallowed*: call a doctor or get medical attention. Do not induce vomiting. Promptly drink a large quantity of milk, egg whites, gelatin solution, or if these are not available, a large quantity of water. Avoid alcohol.

References

Giamberini, L., Czembor, N., and Pihan, J. (1994). "Effects of MEXEL 432 on the settling, detachment and mortality of adult zebra mussels." *Proceedings of The Fourth International Zebra Mussel Conference*, Madison, WI, March 7-10, 1994. University of Wisconsin Sea Grant Institute.

Krueser, R. T., Vanlaer, A., and Damour, A. (1997). "A novel molluscicide, corrosion inhibitor and dispersant," Paper No. 97409, *Proceedings of Corrosion/97*, New Orleans, LA, March 1997. National Association of Corrosion Engineers, Houston, TX, 409/1-409/7.

Mexel S.A (1997a). "Material Safety Data Sheet: MEXEL® 432/0." 5 pp.

Mexel S.A (1997b). "MEXEL 432. Label." 3 pp.

Endothall

The amine salt of the compound endothall, 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid, has been used as a herbicide (HYDROTHOL) against submersed weeds in aquatic environments for a number of years, and its effects on aquatic systems are well understood. It has recently been found to be effective against zebra mussels, and a formulation has been registered as EVAC™ for molluscicidal use.

EVAC™

This formulation of endothall is similar to that of the HYDROTHOL 191 aquatic herbicide. However, only EVAC is registered for molluscicide use (Calgon Corporation 1998b). There is no requirement for a holding period or deactivation prior to discharge.

Chemical Name and Formulations

This formulation has the following characteristics:

a. Active ingredient: Mono(N,N-dimethylalkylamine) salt of endothall (7-oxabicyclo[2.2.1]- heptane-2,3-dicarboxylic acid)

b. Formulation of EVAC biocide:

- 53.0 percent active ingredient, amine salt of endothall (23.36 percent acid equivalent endothall)
- 2 lb technical endothall per gal (240 g per liter)
- Liquid concentrate, soluble in water
- EPA Registration No. 4581-380-10445

c. Source: Calgon Corporation
P.O. Box 1346
Pittsburgh, PA 15230-1346
(412) 777-8000
Health and Environmental Affairs: (412) 494-8000

d. Synonym: TD 2335

Mode of Action

Mussels do not sense this compound in the water and therefore do not close their shells; continued siphoning brings the material into contact with tissues where it acts as a corrosive to membranes, including gills.

It controls established populations of freshwater and saltwater mollusks, and prevents settlement of their immature forms. It also has activity against slime organisms in recirculating systems.

Toxicity is dependent on concentration and exposure time.

Application Strategies

Use this compound periodically for control of established populations in recirculating and once-through cooling water systems. It can be metered directly into the system.

It has potential for treating service water, auxiliary water, wastewater, influent, and fire protection water systems.

Timing of Application

Use when established populations are present.

During breeding and settling season, it can be used to prevent settlement of immature forms of mollusks.

Application Rates and Duration

For established populations in recirculating and once-through cooling water systems, apply at 0.3 to 3.0 ppm of the active ingredient endothall for 6 to 144 hr of exposure. These concentrations are equivalent to 9.1 to 91 L per 38,000,000 L water (2.4 to 24 gal of EVAC per 1,000,000 gal of water).

The higher rates of application and exposure times are required for heavy populations of fouling mollusks and/or with cooler water temperatures (less than 70 EF or 21 EC).

Laboratory studies show that efficacy is dependent on rate of application and time of exposure. For example, treatments of 2.3 ppm for 6 to 7 hr were equivalent to those at 5 ppm for 2 hr.

For byssal thread detachment, use 0.5 mg L^{-1} (Piccirillo, Dionne, and Sandberg 1997).

Posttreatment and Discharge

EVAC does not require a holding period or deactivation after use.

Discharge limits are approximately 50 ppb of amine.

EVAC rapidly dissipates in water. Degradation in the environment is microbial only; it does not hydrolyze or photolyze in an aquatic environment.

Use Restrictions

EVAC can be toxic to fish. Do not discharge effluent containing EVAC into lakes, streams, ponds, estuaries, oceans, or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System permit and notification of the permitting authority in writing prior to discharge.

Do not discharge effluent containing EVAC into sewage systems without previously notifying the local sewage treatment authority. For guidance contact the State Water Board or Regional Office of the U.S. Environmental Protection Agency.

Timing of Effects

Treatment effects are evident within a few days of treatment. Maintain treatment through season.

Toxicological Data

The Signal Word/Toxicity Class is Danger.

This pesticide is toxic to fish.

It is nonpersistent and rapidly degraded by microbes to carbon, hydrogen, and oxygen (Simsiman 1976). It has short persistence and does not bioaccumulate, does not form toxic metabolites, or require detoxification. It has low to intermediate nontarget toxicity at regular application rates, as listed in the following tabulation.

Toxicity of Dimethylalkylamine Endothall

Species	Conditions	Exposure Period, hr	Acute Toxicity, Median Lethal Concentration, mg L^{-1}
Largemouth bass	Static	96	0.1-0.3[1]
Bluegill sunfish	Static	48	0.8
		96	0.06-0.2[1]
Redear sunfish	Static	96	0.1-0.2[1]
Golden shiner	Flow-through	120	0.32-1.6

Note: Data from Calgon Corporation (1998a).
[1] Diamine salt.

Precautions

EVAC is a concentrated product. It is fatal if absorbed through skin, and may be fatal if swallowed or inhaled.

It is corrosive, causing irreversible eye damage and skin burns. Do not get in eyes, on skin, or on clothing.

Wear the following personal protective equipment for concentrated product: coveralls over long-sleeved shirt and long pants; waterproof gloves; protective eyewear; chemical-resistant footwear plus socks.

Field Instructions and Guidance on Operational Applications

Suspension of the blowdown from cooling towers for 6-12 hr after application and/or segmenting plantwide treatments will reduce the level of product remaining in the effluent.

This product appears to be most effective at temperatures greater than 63 EF (17 EC). At temperatures of between 63 and 54 EF (17 and 12 EC), the mortality of zebra mussels exposed to 3.0 mg L^{-1} (3 ppm) for 8 hr was approximately 20 percent compared with 80 percent mortality among zebra mussels exposed under the same conditions, but at a temperature of 68 EF (20 EC) (Piccirillo, Dionne, and Sandberg 1997).

Adjuvant or Deactivant Use

No deactivant is required.

Application Techniques

EVAC can be metered directly into the system with a positive displacement pump or into a flow of dilution water for use in a distribution header. One continuous application should be made at a convenient point of uniform mixing, such as a basin area, pump area, or other reservoir or collecting area from which treated water will be circulated uniformly throughout the system.

Antidote Information

Use the following antidotes:

a. *If swallowed*: call a physician or Poison Control Center. Have the victim drink 1 or 2 glasses of water and induce vomiting, unless person is unconscious.

b. *If on skin*: wash with plenty of soap and water. Get medical attention.

c. *If in eyes*: hold eyelids open and flush with water for 15 min. Get medical attention.

d. *If inhaled*: remove victim to fresh air. Get medical attention.

e. *Note to physician*: probable mucosal damage may contraindicate use of gastric lavage. Measures against circulatory shock, respiratory depression, and convulsion may be needed.

f. *Emergency phone number*:

(1) Calgon Corporation: (412) 494-8000

(2) CHEMTREC: 1-800-424-9300

References

Calgon Corporation. (1998a). "EVAC™ Biocide. Material Safety Data Sheet," Pittsburgh, PA.

Calgon Corporation. (1998b). "EVAC™ Biocide. Registered label," Pittsburgh, PA.

Piccirillo, V. J., Dionne, E., and Sandberg, G. (1997). "TD 2335: Laboratory and field efficacy studies for control of zebra mussels in electric power plants," *Zebra mussel and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 534-540.

Simsiman, G. V. (1976). "Diquat and endothall: Their fates in the environment," *Residue Reviews* 62, 131-74.

Metals and Their Salts

Both copper and a range of potassium salts have been shown to have activity against zebra mussels. Their low toxicity to other organisms in water and long history of use in water treatment make them potential solutions for a range of problem zebra mussel sites and systems.

Copper Ions

The presence of excess copper ions in water is inimical to a number of aquatic organisms, including algae, plants, mussels and clams, and has a long history of use in marine antifouling coatings. McMahon and Tsou (1990) note that copper is relatively lethal to zebra mussels, with 5 ppm copper ions for 24 hr giving 100 percent kill of veligers.

MacroTech

The commercial MacroTech ZM-Series devices employ copper and aluminum anodes to supply copper ions to water at a low but toxic level. The gelatinous nature of the aluminum hydroxide formed enhances flocculation and deposition of the copper ion on surfaces, which then makes them unacceptable for settling (Blume, Fraleigh, and Van Cott 1994; Race 1995; Blume and Fitzgerald 1996).

Chemical Name and Commercial Formulations

The MacroTech technique uses these compounds:

a. Chemical:

- Copper ions (Cu^{++}) and Aluminum (Al)
- Aluminum hydroxide ($Al(OH)^3$)

b. *Source*: MacroTech, Inc.
246 Mamaroneck Road
Scarsdale, NY 10583-7242
(914) 723-6185
wjblume@prodigy.net

Mode of Action

Incoming water is treated with copper and aluminum by the controlled electrolytic dissolution of anodes of these materials to produce copper ions and aluminum hydroxide. Presence of copper in water inhibits veliger activity and development through direct toxicity. The aluminum hydroxide has a flocculent

activity that aids in precipitating veligers, and it forms an anodic, fluid film on surfaces, which acts as an antifouling coating to inhibit biofilm formation and postveliger settlement. This activity also reduces potential for microbiologically induced corrosion.

Application Strategies

Deploy this device to treat incoming water in flow-through or recirculating service water systems.

Timing of Application

To prevent settlement at the postveliger stage, apply on a continuous basis during the reproductive and settling season.

Application Rates

The MacroTech unit maintains copper ions at 5 to 10 ppb above ambient levels.

Maximum Water Concentration

Currently the U.S. Environmental Protection Agency Drinking Water Standard for copper is a maximum contaminant level of 1.3 mg L^{-1} or 1,300 ppb (U.S. Environmental Protection Agency 1991).

No detoxification is required. Water can be discharged to potable water without detoxification.

Use Restrictions

Copper concentration should not exceed 1 mg L^{-1} (potable water) by weight copper.

Currently, there are no restrictions on the use of treated water immediately following treatment.

Timing and Appearance of Effects

Copper is relatively lethal to zebra mussel; 5 ppm Cu for 24 hr has been shown to give 100 percent kill (McMahon and Tsou 1990).

Toxicological Data

The Signal Word is Caution.

Copper toxicity to aquatic organisms can vary with water hardness, as shown in the following tabulation.

Toxicity of Copper to Aquatic Organisms				
Species	**Chemical**	**Hardness mg L^{-1} as $CaCO_3$**	**Exposure Period, hr**	**Acute Toxicity, Median Lethal Concentration, mg L^{-1}**
Cutthroat trout	Copper chloride	18-205	96	15.7-367
Rainbow trout	Copper chloride	42-194	96	57-574
White perch	Copper nitrate	53	96	6,200
Striped bass	Copper nitrate	53-55	96	4,000-4,300
Bluegill sunfish	Copper chloride	43	96	1,250
Largemouth bass	Copper nitrate	100	96	6,970

Precautions

Monitor water hardness and presence of desirable aquatic species in outfall water.

Exposure to copper may produce skin and gastrointestinal irritation.

Field Instructions and Guidance on Operational Applications

Install MacroTech treatment unit as directed by manufacturer.

Adjuvant/Detoxicant/Deactivant Use

None is required.

Application Techniques

A side stream of fresh water is passed through the MacroTech treatment unit, wherein a copper and aluminum concentrate is formed. The treated water is then reinjected to the intake for final dilution and the treatment of the entire system.

Antidote Information

Wash contacted areas. Get medical attention if irritation persists.

References

Blume, W. J., and Fitzgerald, W. T. (1996). "Field experience with copper ions and aluminum floc for preventing settlement of zebra mussels and Asiatic clams." *Proceedings of the Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 5-7, 1996.

Blume, W. J., Fraleigh, P. C., and Van Cott, W. R. (1994). "Evaluation of copper ions and aluminum floc for preventing settlement of zebra mussels." *Proceedings of the Fourth International Zebra Mussel Conference*, Madison, WI, March 1994.

McMahon, R. F., and Tsou, J. L. (1990). "Impact of European zebra mussel infestation to the electric power industry." *Annual Meeting, American Power Conference*, Chicago, IL, April 1990, 9 pp.

Race, T. (1995). "Copper-based marine antifoulants," Technical Note ZMR-2-02, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS, 2 pp.

Race, T. D., and Kelly, M. A. (1997). "A summary of a three year evaluation effort of anti-zebra mussel coatings and materials." *Zebra mussels and aquatic nuisance species*. F. M. D'Itri, ed., Ann Arbor Press, Chelsea, MI, 359-388.

U.S. Environmental Protection Agency. (1991). "The National Primary Drinking Water Regulations (NPDWR) for Lead and Copper." 40 CFR Parts 401 and 142.

Potassium Compounds

The K^+ ions of potassium compounds have some selective activity, being highly toxic to zebra mussels, where they interfere with membrane integrity and respiration (Fisher et al. 1991; Claudi and Mackie 1994), but not affecting fish (Waller et al. 1993). While too harmful to native mussels to have gained approval for use in once-through systems, potassium is suitable for contained or closed-loop systems, or those such as fire protection systems where outfall of large amounts of concentrated solutions is not a problem (Claudi and Mackie 1994; Lewis et al. 1996). A method for treating such semistatic systems with the effects of potassium ions (K+), using commercially available potash has been developed by Aquatic Sciences, Inc., Canada. As an unregulated, low-cost product, potash has been shown to be acutely toxic to mussels under both warmwater and coldwater conditions (>5 ºC). At relatively low levels, potassium also appears to be a selective molluscicide. Since potash is regularly used as a fertilizer in agriculture, it is widely available and generally regarded as safe.

Chemical Name and Commercial Formulations

Potassium is available from various sources under the following chemical names:

a. Potassium phosphate, monobasic, KH_2PO_4

b. Potassium chloride, KCl

c. Potash

Mode of Action

Potassium causes several changes in zebra mussels, including prevention of valve closure, reduction in filtration rate, and mortality (Wildridge et al. 1996). It is thought to kill adult mussels by destroying the membrane integrity of the gill epithelium, thus eliminating the ability to respire (Fisher et al. 1991).

Application Strategies

Deliver a potassium-rich solution to water to be treated.

Timing of Application

Treatment can be at any time of year, as potash has been found to be efficacious at temperatures as low as 5 ºC (40 ºF).

Application Rates

Tests of various potassium salts show that adults are killed rapidly, with median lethal concentrations LC_{50}'s at 80 to 313 mg L^{-1} (Fisher et al. 1991). A concentration of 50 mg L^{-1} prevents settlement (Fisher, Fisher, and Polizotto 1993). The following tabulation lists the toxicities of potassium salts.

Toxicity of Potassium Salts to Zebra Mussel

Potassium Salt	LC_{50}, mg L^{-1}	Reference
KCl	150 (48-hr) 138	Waller et al. 1993 Fisher et al. 1991
KH_2PO_4	92	Fisher et al. 1991
$K_4P_2O_7$ (potassium pyrophosphate)	94	Fisher et al. 1991

To prevent primary settlement, use 30 ppm on a continuous basis.

At water temperatures above 15 ºC, 40 ppm for 2 weeks gives control.

At water temperatures above 15 ºC, 100 ppm gives 100 percent mortality in 48 hr.

Maximum Water Concentration

Check with State agencies to see if a National Pollutant Discharge Elimination System permit is required for facility-specific discharge.

Use Restrictions

Restrict concentrations to maintain permitted discharge limits.

Timing and Appearance of Effects

Treatment effects are seen within 24 hours. Maintain treatment for effective control.

Toxicological Data

The following tabulation lists toxicities of potassium chloride.

Toxicity of KCl to Aquatic Organisms		
Organism	**Condition**	**No-Observed-Effect Level, mg L^{-1}**
Daphnia magna		>100
Gambusia affinis		>186
Helisoma spp.		>186
Anondonta imbecillus	With sediment Without sediment	>100 LC_{50} 76
Ceriodaphnia dubia		>100
Fathead minnows		>100
Rainbow trout		>100
Note: Data from Fisher et al. 1991.		

Precautions

These compounds may cause irritation during use. Avoid contact with eyes, skin, clothing, and wash them thoroughly after handling.

Field Instructions and Guidance on Operational Applications

Use biobox monitors to assess effectiveness of treatment.

Discharge to ground or to greater volumes of water.

Adjuvant or Detoxicant Use

No adjuvant or detoxification is required.

Application Techniques

Meter solution into water system.

Antidote Information

Use the following antidotes:

a. *Ingestion*: if swallowed and the person is conscious, immediately give large amounts of water. Get medical attention.

b. *Inhalation*: if a person breathes in large amounts, move the exposed person to fresh air. Get medical attention.

c. *Eye contact*: immediately flush with plenty of water for at least 15 minutes. Get medical attention.

d. *Skin contact*: immediately wash with plenty of soap and water for at least 15 minutes.

References

Claudi, R., and Mackie, G. L. (1994). *Practical manual for zebra mussel monitoring and control*. Lewis Publishers, Boca Raton, FL, 227 pp.

Fisher, S. A., Fisher, S. W., and Polizotto, K. R. (1993). "Field tests of the molluscistatic activity of potassium chloride on zebra mussel veligers." *Third International Zebra Mussel Conference, Agenda and Abstracts*, Toronto, Canada, February 23-26, 1993.

Fisher, S.W., Stromberg, P., Bruner, K. A., and Boulet, L. D. (1991). "Molluscicidal activity of potassium to the zebra mussel, *Dreissena polymorpha*: Toxicity and mode of action," *Aquatic Toxicology* 20, 219-234.

Lewis, D. P., Piontkowski, J., Neuhauser, E., and Knowlton, J. (1996). "Chronic exposure of adult and larval zebra mussels at low level potassium concentrations: Laboratory studies." Abstract from the *Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 5-7, 1996. Sea Grant Institute, University of Wisconsin, Madison, WI.

Waller, D. L., Rach, J. J., Cope, W. G., Marking, L. L., Fisher, S. W., and Dabrowski, H. (1993). "Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms," *J. Great Lakes Res.* 19, 695-702.

Wildridge, P. J., Werner, R. G., Doherty, F. G., and Neuhauser, E. F. (1996). "Toxicity of potassium to adult zebra mussels." Abstract from the *Sixth International Zebra Mussel and Other Aquatic Nuisance Species Conference*, Dearborn, MI, March 5-7, 996. Sea Grant Institute, University of Wisconsin, Madison, WI.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2000	Final report

4. TITLE AND SUBTITLE
Zebra Mussel Chemical Control Guide

5. FUNDING NUMBERS
WU 33156

6. AUTHOR(S)
Susan L. Sprecher, Kurt D. Getsinger

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Engineer Research and Development Center
Environmental Laboratory
3909 Halls Ferry Road, Vicksburg, MS 39180-6199

8. PERFORMING ORGANIZATION REPORT NUMBER
ERDC/EL TR-00-1

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Corps of Engineers
Washington, DC 20314-1000

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 words)***

Control and prevention of macrofouling caused by the freshwater zebra mussel, *Dreissena polymorpha* (Pallas), is a major concern of managers of inland waterways, water treatment plants, and power generation facilities in many areas of eastern North America. The threat to structures in navigable waterways makes the issue of interest to the U.S. Army Corps of Engineers.

In North America, chemical applications to water have been the most commonly used method of zebra mussel treatment and control for internal and closed systems. Numerous organic and inorganic chemicals with toxicity to zebra mussels can provide versatile, cost-effective, and easy to implement ways to deal with established populations, and can prevent new infestations. Chemical treatments can be designed to protect whole systems very safely when use and discharge are carried out in compliance with environmental regulations.

This report describes basic guidelines for the use of molluscicidal compounds that are currently registered with the U.S. Environmental Protection Agency for zebra mussel control.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Chemical control	Molluscicides	114
Dreissena polymorpha	Zebra mussel	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED		

APPENDIX 5

Compilation lab and biobox trial data for MOI 401-Pf CL145A.

Appendix. MOI 401 Efficacy Trials

Jar Testing

To confirm efficacy of MOI 401 produced after each fermentation run and product formulation mussel bioassays are performed at the MOI laboratories in Davis, California. MOI 401 is applied to the desired concentration in 250 mls of water and held for a 24 hour period. Mussels are pulled from storage aquariums at a minimum of 24 hours prior to applying MOI 401 to check for mussel health and siphoning. A minimum of three replicates are prepared per treatment scenario with 20 mussels per jar. A positive control of product from previously tested material is included in the treated jar setup to compare the efficacy of new fermentations and formulations to previously identified effective product. Triplicate untreated controls are also prepared with the same set of mussels. Jars are aerated 24 hours prior to treatment and during the 24 hour treatment. After treatment the water is changed daily and each group of mussels are checked for dead mussels daily for 10 days. All bioassays are conducted at room temperature.

To conduct the bioassay trials, quagga mussels are harvested from infested water ways, sorted and packaged, and shipped to the MOI laboratory. Upon receipt mussels are placed into aquariums for storage prior to trials. In order to obtain preliminary numbers and efficacy of product on Colorado River mussels, mussels have been collected from Lake Havasu Bullhead City, AZ, near Davis Dam.

A summary of the 10 day mortality of recent bioassay results are presented in the Table 1, below.

Table 1. Summary of Selected Bioassay results of MOI 401

Date	Dose (ppm)	Mortality (%)	Std. Deviation (+/- %)	Mussels
4-Dec-08	200	66.7	19	Quaggas, NY
	0	0	0	
16-Dec-08	200	100	0	Quaggas, AZ
	0	0	0	
9-Mar-09	200	100	0	Quaggas, AZ
	200	96.67	5.77	
	100	93.67	5.77	
	100	83.33	15.28	
	0	10	10	

All jar assay results reported in the table above had less than 10 percent mortality in the untreated control. Bioassay tests in which the mortality is greater than 10 percent in the untreated controls are considered invalid and tests are rerun.

Jar assays are not directly representative of results that would be observed in the field since they do not represent a flow through or ideal mussel environment. In addition, mussel health is altered during

Jar assays are not directly representative of results that would be observed in the field since they do not represent a flow through or ideal mussel environment. In addition, mussel health is altered during shipment and storage in the laboratory. The ideal situation to test the efficacy of the MOI 401 product is conducting flow through biobox trials at a site in which mussels have already infested all local source water. Jar bioassays in the lab provide MOI a method to test fermentation and formulation optimizations and to select product improvements that should be tested in the field and remain a critical component of preliminary product efficacy testing.

Flow through biobox trials

Flow through biobox trials are being conducted at the United States Bureau of Reclamation, Davis Dam, Bullhead City, Arizona because mussels have already impacted the operations of the USBR Dams on the Colorado River. Mussels are collected on the Colorado River near Bullhead City, AZ at Katherine Landing which is a short distance upriver of Davis Dam. Mussels are immediately brought back to Davis Dam to be checked for siphoning (indication of feeding) and other behaviors indicating that they are healthy mussels before they are sorted for size and placed in small acrylic pipe sections. Triplicate pipe sections containing 50 mussels each are then placed in one of the three flow through bioboxes connected to Davis Dam cooling water system. One biobox serves as the untreated control for the trials and the other two are used for treatment with MOI 401. The flow rate is individually regulated for each of the bioboxes with ball valves and measured using a graduated cylinder and a stop watch. All biobox treatments are currently conducted at 1 gallon per minute. MOI 401 is applied into the bioboxes using a peristaltic pump at pre-determined rates to achieve desired application concentrations. Applied concentration is confirmed by measuring optical density or turbidity. After treatments, mussels remain in the bioboxes under flow through conditions. Mussel mortality is scored periodically.

Table 2 presents a summary of the biobox trials that have been conducted to date at Davis Dam and the total mussel mortality as scored at this time. Biobox trials were conducted the weeks of January 12, February 23, and March 23. The water temperature listed in the table below is the temperature of the Colorado River water in the bioboxes when MOI 401 was applied. After the trial mussels remained in cleaned bioboxes that receive the same Colorado River water; therefore, as the Colorado River water temperature increases the water temperature that the mussels see also increases.

Table 2. Summary of Biobox trials conducted at Davis Dam and final mussel mortality counts

Trial Week and Water Temperature (degree C)	Dose (mg/L)	Treatment Time (hours)	Percent Mortality (%)	Std. dev. (+/- %)	Mortality Check (day)
January 12, 2009 10.5 deg. C	200	6	60	5	49
			70	4	51
February 23, 2009 12 deg. C	200	6	64	8	47
	100	4	63	2	47
	100	6	67	4	47
March 23, 2009[2] New Formulation 14 deg. C	100	6	87	3	34
	100	6	83	6	33
	50		84	2	33
	75	4	82	4	32
	75	6	82	5	32
	25	4	63	9	32
	25	6	75	6	32

[1]Initial Temperature refers to the temperature at which MOI 401 was applied; however, the temperature on the Colorado River is currently gradually rising

[2]March trials mortality checks are still on-going as of April 15, 2009

The figures below present mussel mortality over time for the trials presented in the table.

Figure 1 presents percent mussel mortality over time, in days, at 200 ppm and 100 ppm of MOI 401 at 10.5, 12, and 14 deg. C. March trials, at 14 deg. C were conducted with a new formulation of MOI 401 that provided for a more uniformly dispersed media. There was no significant difference in the trial results observed in January and February at 100 ppm and 200 ppm. It is unlikely that all of the increase in mussel mortality can be attributed to the increase in temperature since no increase in mussel mortality was observed between 10.5 and 12 deg. C. Previous research conducted by NYSM has demonstrated that temperature significantly impacts mussel mortality and some of the increase in mortality is likely due to the overall increase in temperature from 10.5 deg to 14 deg C. With the initial formulation a decrease in efficacy was not observed when trials were conducted at 100 ppm rather than 200 ppm.

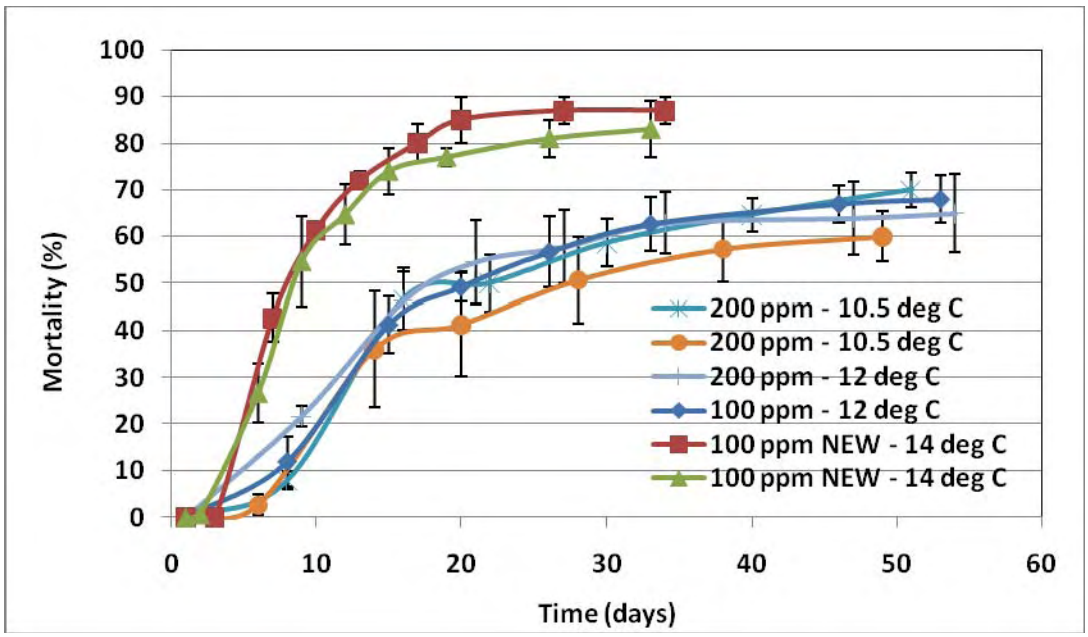


Figure 1. Mussel moratatliy over time for trials conducted at 100 and 200 ppm, with an application time of 6 hours, and conducted in water at 10.5, 12, and 14 deg C. "New" in the legend denotes new MOI 401 formulation.

In March, all biobox trials were conducted with a new formulation which also resulted in a more dispersed and well mixed product. Colorado River water temperature in the flow through bioboxes was 14 deg C during the trials. MOI 401 was applied for 6 hours at 25, 50, 75 and 100 ppm (Figure 2). When compared to 100 ppm, 50 and 75 ppm doses resulted in similar mussel mortality. Decreasing the dose to 25 ppm did result in a significant decrease in mussel mortality compared to treatments at 100 ppm with this formulation in water at 14 deg C.

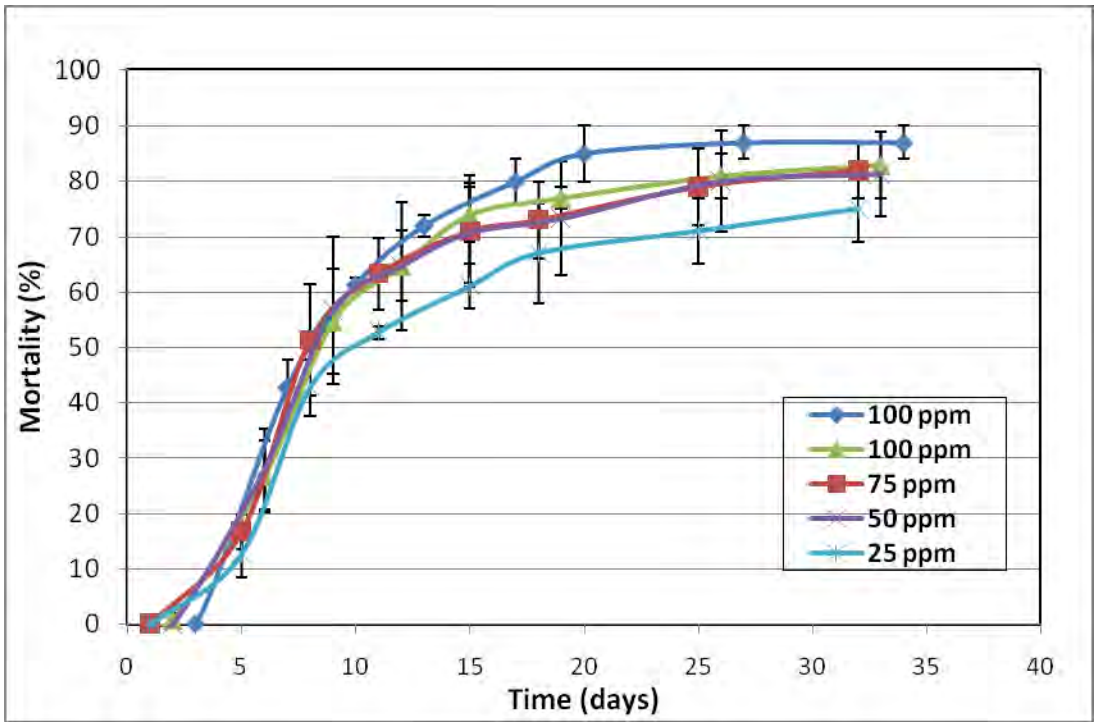


Figure 2. Mussel morality over time for trials conducted at 25, 50, 75, and 100 ppm, with a 6 hour application time, and in 14 deg C water with new MOI 401 formulation.

MOI 401 was applied at 4 and 6 hour treatment application times during the March trials at doses of 25 and 75 ppm (Figure 3). For both concentrations a decrease in mortality was observed when mussels were exposed to only 4 hours of treatment compared to 6 hours.

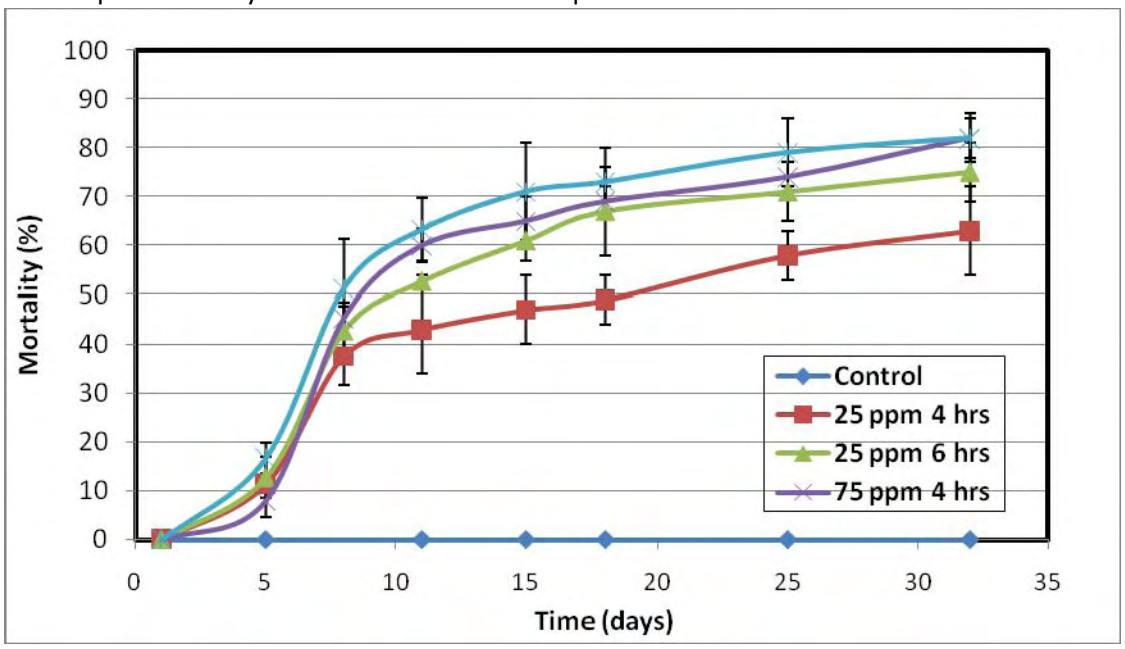


Figure 3. Mussel morality over time for trials conducted at 25 and 75 ppm, in 14 deg C water, and at application rates of 4 and 6 hours with a new MOI 401 formulation.

APPENDIX 6

Reclamation letter to MBI informing intent to pursue Section 18.

United States Department of the Interior

BUREAU OF RECLAMATION
PO Box 25007
Denver, Colorado 80225-0007

IN REPLY REFER TO:

86-69000
PRJ-1.10

AUG 19 2009

Ms. Pamela Marrone, PhD., CEO
Marrone Bio Innovations, Inc.
2121 Second St. Suite 107B
Davis, CA 95618

Subject: Section 18 Exemption of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) for MOI 401

Dear Dr. Marrone:

We are presently working jointly with your firm to evaluate a bacterial product of *Pseudomonas flourescens* (MOI 401) as a means to control quagga mussels at Davis Dam on the Colorado River near Laughlin, Nevada. We understand that you are pursuing registration of MOI 401 (Zequanox™) as an organic pesticide for mussel control under Section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and that this process may require as much as 12 to 18 months to be completed.

Section 18 of FIFRA authorizes the Environmental Protection Agency (EPA) to allow limited use of a pesticide if EPA determines that an emergency condition exists, and we are pursuing such an exemption for use of MOI 401 at our Lower Colorado River Dams.

For further discussions and coordination on this topic, please contact Mr. Fred Nibling in our Denver Office at 303-445-2202 or fnibling@usbr.gov.

Sincerely,

Curtis A. Brown, Ph.D.
Director, Research Office

cc: Mr. Fred Nibling, Research Botanist
Environmental Applications
and Research Group
Bureau of Reclamation
P.O. Box 25007, MS 86-68220
Denver, CO 80225

APPENDIX 7

Photographs of mussel infestation in facilities.

Appendix 7

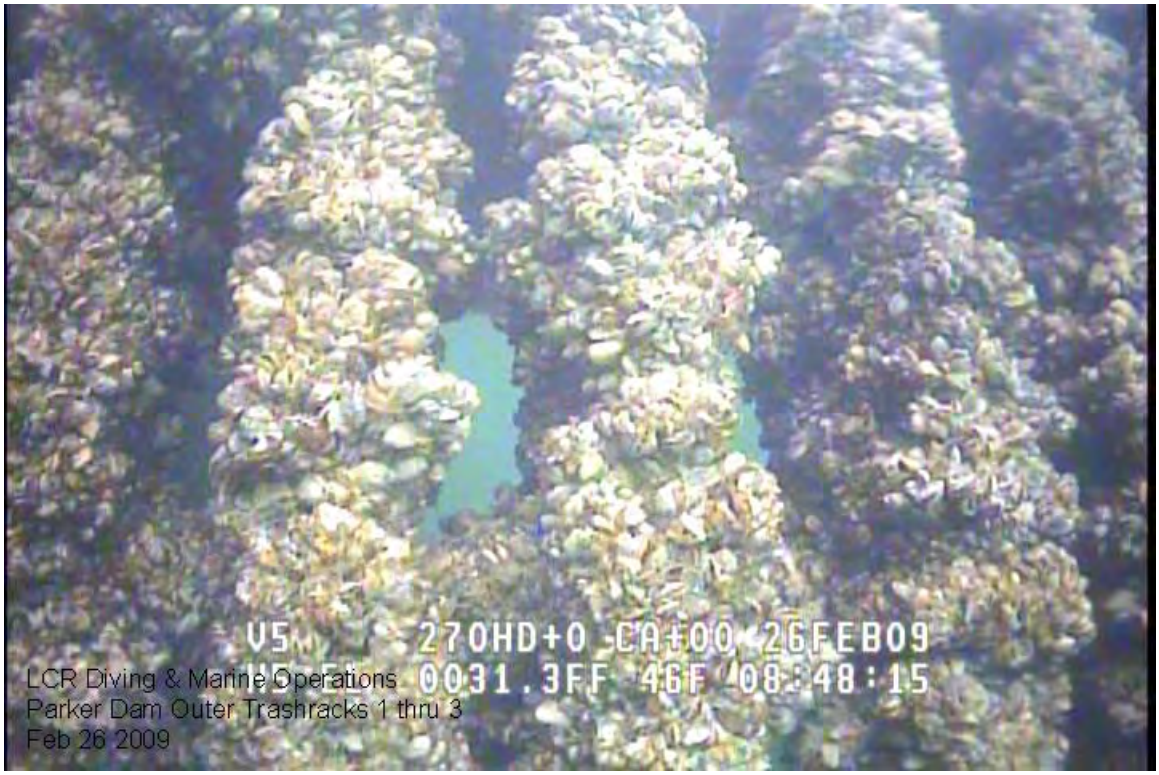


Figure 1. Quagga mussels on trashracks at Parker Dam.

Figure 2. Quagga mussels on intake trashracks at Hoover Dam.

Figure 3. Clogged pipe.

Figure 2. Dead mussel debris in cooling unit.

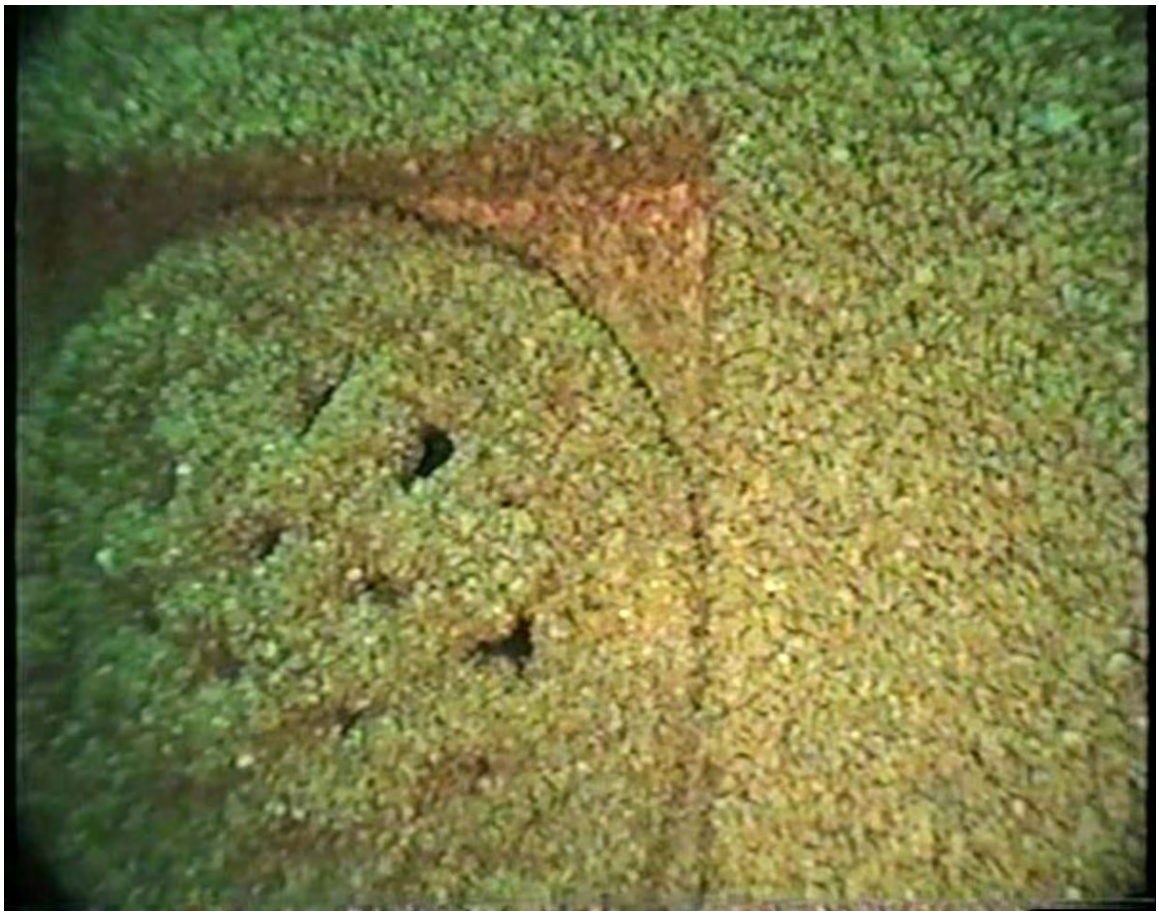


Figure 6. North domestic water intake.

Figure 7. Unit 1 turbine bearing oil heat exchanger Y strainer.

Figure 8. Unit 1 turbine bearing oil heat exchanger, holes are 7/16”.

Figure 9. Old style strainer basket pulled from service for inspection.

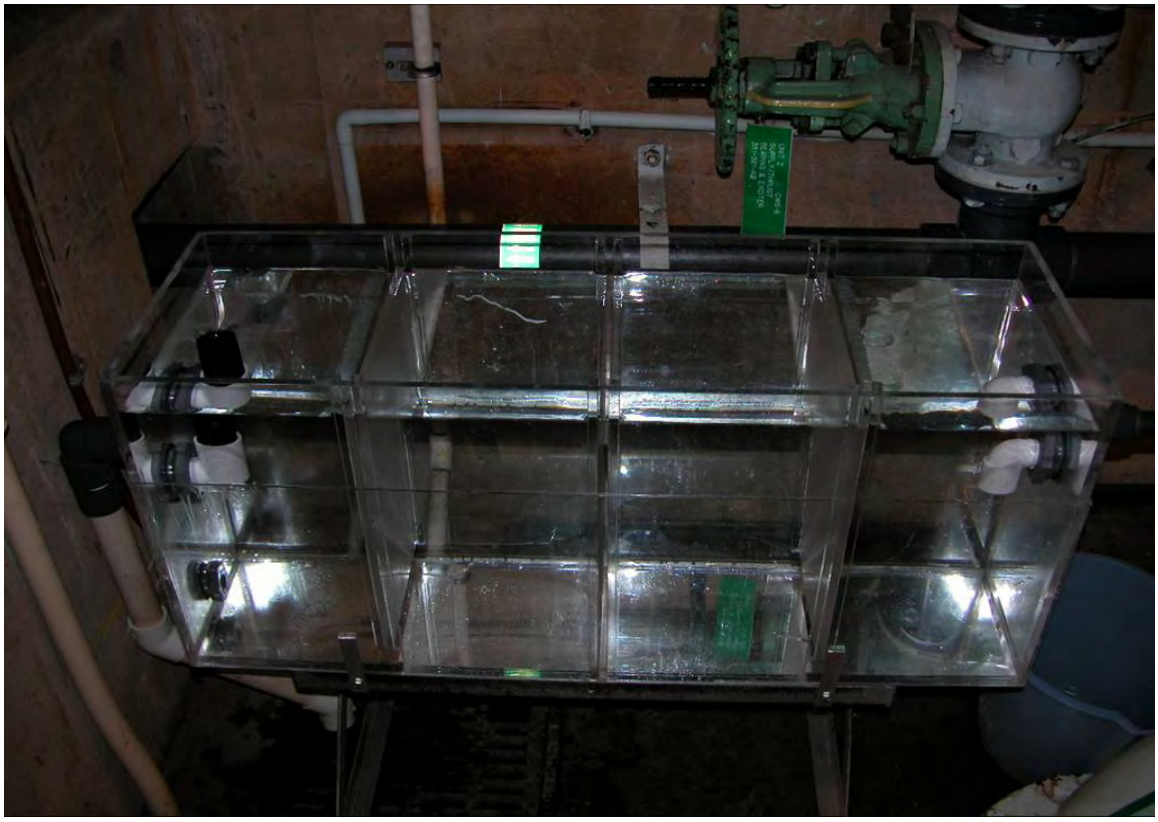


Figure 10. Unit 2 cooling water intake, quagga monitoring tank. Installed 3/11/08.

Figure 11. Unit 2 cooling water intake, quagga monitoring tank, 6/26/08

Figure 12. 8" cooling water supply line on Hoover Dam Generator (Nevada #8), November 2008.

APPENDIX 8

Experimental Use Permit (# AZ 09-001), Arizona Department of Agriculture.

JANET NAPOLITANO
Governor

DONALD BUTLER
Director

Arizona Department of Agriculture

1688 W. Adams Street, Phoenix, Arizona 85007
(602) 542-3578 FAX (602) 542-0466

December 12, 2008

Keith Pitts
2121 Second St., Ste. B-107
Davis, CA 95618

EUP Permit # AZ 09-001

Dear Mr. Pitts,

This letter acts as final approval for an Experimental Use Permit (EUP) in Arizona for:

Pseudomonas fluorescens CL 145A, EUP **# AZ 09-001**

Your product, Pseudomonas fluorescens CL 145A, is registered for experimental use in Arizona on quagga mussels. This permit applies only to the two tests discussed at our meeting on September 25, 2008. The first is the test designed to use the bioboxs. Since this is an experimental use, we require that all treated water be disposed of in the manner we discussed, where the treated water is held in the holding tank and then pumped to the settling pond and then land filled. This will prevent contact of any kind with humans or animals.

The second test is in the 10 inch domestic water supply pipe and can only proceed when the ability exists to close off the intake side of the line upon introduction of the appropriate amount of Pseudomonas fluorescens CL 145A. Since this is an experimental use, we require you to follow the same procedure as above for the bioboxs experiment in disposing of the treated water.

This permit, and the product registration, will expire on December 31, 2009. Should you need to continue testing beyond that time, you will need to request renewals of your registration and the EUP.

For monitoring purposes, the applicator is to notify the agency by calling 1-800-423-8876 at least 24 hours in advance with the exact time, date and location of the application. This number is the pesticide hotline answering machine and all calls are logged.

Thank you for your cooperation,

Gary Christian

Licensing Program Manager
602-542-0903

Appendix C

Treatment Evaluation Methodology

Treatment Evaluation Methodology

The scientific methodologies employed to determine the potential for veliger (i.e., juvenile mussel) settlement in the cooling water subsystem and to evaluate the effectiveness of the settlement prevention treatments are described here. Quantification of the veliger population (and specifically those veligers in the age class likely to settle) are conducted in the waters feeding into the dam, into the cooling water system, and coming through the system to the outlet of the cooling water system. These populations are correlated to observe newly settled mussels on settlement plates placed in the monitoring bioboxes.

Settlement Monitoring Biobox Locations

Two bioboxes have been placed at the inlet on each cooling water subsystem (control and treated) and at the outlet of each cooling water subsystem (control and treated) for a total of 8 bioboxes. The location of the bioboxes on the outlet of the cooling water subsystems is just before the water is released back into the Colorado River at the tailrace.

Settlement prevention will be evaluated by placing numerous identical square pieces of material (plates) within the bioboxes before the treatment begins, and then removing some of the plates periodically to check for newly settled mussels on the plates. The difference between mussel settlement on the plates in untreated control bio boxes will be statistically compared with the settlement on the plates in the treated bio boxes.

Biobox Settlement Plates

While mussels will settle on almost any substrate (with the exception of copper) certain substrates have higher settlement rates than others. In this treatment, PVC plates will be used based on the ease of cutting the material and the availability of the material to Reclamation. These plates will be placed into PVC holders in the biobox chambers. These plate holders will maintain consistent plate orientation facing into the flow coming through the bioboxes throughout the treatment. These plates are removed and assessed every two weeks, to evaluate pediveliger settlement.

The dimensions of each settlement plate will be 10 x 10 centimeters (cm). This will allow for good flow around the plates within the biobox compartment, balanced with a reasonable surface area per plate for settlement. Settlement plates will be as thin as possible, so settlement area can be determined with minimal consideration of area along the edge of the plate, and error can be minimized when scraping the plate (i.e. missing settled mussels on the edge, or accidentally scraping mussels attached to the edge). Prior to placement in a biobox, settlement plates will be placed in 30 µm filtered (veliger free) aerated raw river water for two weeks to form biofilm, a microscopic coating that some hypothesize is required for mussel attachment.

Plates will be placed in a holder with angled slots (consistent in size and depth, exactly ½ the depth of the PVC pipe) cut into them that will hold plates in a slightly off vertical (~15°) slanted

orientation, with a 2 cm distance between each plate to minimize flow impact. The plate holder will also keep the plates raised from the floor of the biobox. The edge of the plates will be orientated facing into the flow, with the flat face of the plate facing the acrylic biobox wall. For stability, each holder will have two slotted supports on the bottom and on the top of each plate (See example diagrams: Figures A.1 - A.3 below). Each holder will contain 14 plates, 12 for sampling. The two outside plates will not be used for sampling because of their different/inconsistent environment. The top sections of the holder will be easily detachable so a plate can be grasped and removed without disturbing any other plates and settled mussels.

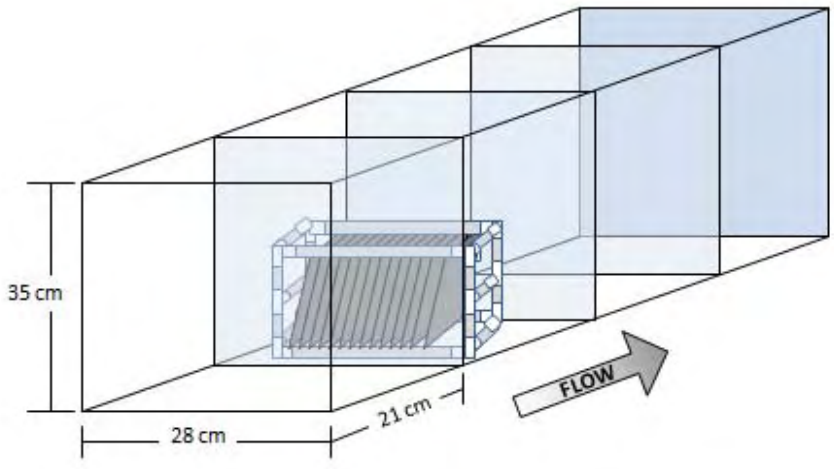


Figure A.1. Orientation of Settlement Plates and Settlement Plate Holder within a Biobox Compartment.

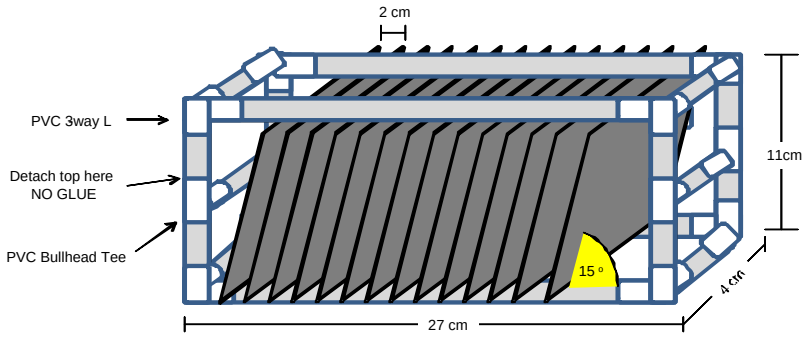


Figure A.2 Orientation of Settlement Plates within a Holder, with 14 total plates, at a 15° off vertical slant.

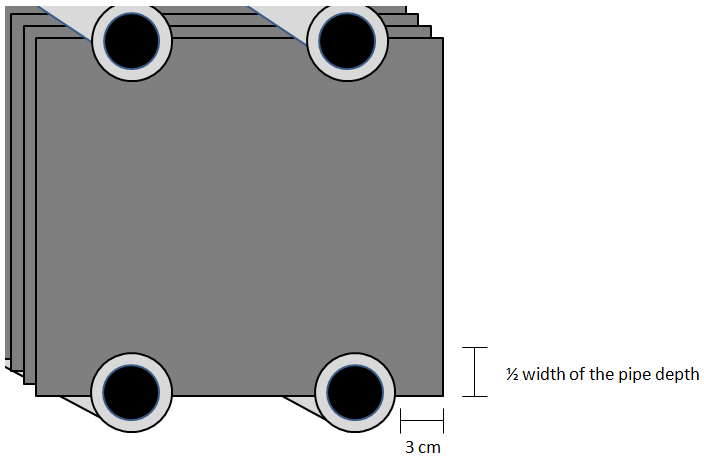


Figure A.3 Settlement Plates will be held within the Plate Holder by slots cut (at the 15° angle) halfway through the PVC piping.

One holder of plates will be placed in each of the two center biobox compartments. The upstream compartment will contain a holder filled with plates (after biofilm development), placed at the beginning of a treatment. The second compartment (downstream) will contain plates placed a minimum of two weeks prior to commencement, and can be used to evaluate

initial treatment efficacy at removing two week old settlement. Hydrologists from the Technical Services Center (TSC) of the Bureau of Reclamation are evaluating exact plate orientation and biobox placement to determine ideal flow pattern and exposure to settlement plates.

Veliger Density and Size Classification Monitoring

Water samples will be collected to determine veliger density and size classification at three locations. Quantitative assessment of the concentration of veligers will be completed within the forebay directly preceding the dam, within the water flowing through the pipelines leading into the bioboxes (both control and treated) before treatment, and also down line of the treatment. This assessment will measure the potential for mussel settlement within the cooling water subsystems and will provide reference to other future users on the conditions of this application. Collection of water parameter metadata will also occur, including temperature, pH, D.O., time of day, salinity, and either secchi depth (open water) or turbidity (biobox).

Every two weeks, plankton tow samples will be collected and sorted to determine veliger density and size classification in the Davis Dam forebay. Samples collected within the dam from the biobox inlets will be collected with previously published biobox veliger collection methods, from a valve at the inlet of the bioboxes (Claudi and Mackie, 1994). Samples will be stored on ice and will be evaluated within 3 days or preserved for future evaluation.

Veliger enumeration will be conducted using the Standard Method 10200 G and the United States Army Corps of Engineers (USACE) methodology for veliger enumeration (USACE, 2002). The guidelines developed by the USACE will also be used for identification of the life stages of quagga mussels, and to avoid mis-identification of quagga veligers.

Settlement Assessment

Quantitative assessment of the concentration of recently settled veligers on replicate settlement plates within the bioboxes will be performed every two weeks from each biobox (8 boxes total) throughout the treatment. Subsections of the plates will be photographed with a digital microscope, and the images will be assessed for veliger settlement. This method will be validated against the traditional method where plates are scraped and mussels identified from the scraped material with cross polarized microscopy (Johnson, 1995).

Plates will be collected on Day 13 and 27 of the month to analyze settlement at the longest time point after the previous dose, but still one day prior to next dose to avoid compounding effects of physically disturbing recently settled mussels combined with product treatment. If after two weeks (Day 13 plate check), treatment has been successful and no settlement has been observed, the same treatment will be continued and monitored again at the one month time point (Day 27) for final confirmation of dose efficacy. If settlement prevention has been successful, the dosage will be decreased to the next lower increment. If at the Day 13 check, settlement has occurred, treatment will still continue, however, if at Day 27 settlement still is occurring, the dose and time may be increased.

Assessment of Live vs. Dead Settled Mussels

When observing mussels in any life stage, it is hard to determine if a mussel is dead, or is simply closed and not moving. Mortality of mussels can be assessed if mussel shells are gaping open and do not close or respond to stimulus (prodding) or if there is no tissue attached inside the gaping shell. During assessment of settlement plates, a high concentration (compared to control) of recently settled mussel shells devoid of any tissue or unresponsive to stimulus would indicate settled mussel mortality. That is, recently settled mussels are effectively killed by the treatment, but their shells aren't detached from the settlement plate yet because the bysal thread attachment hasn't decomposed yet. If this is observed, plates will be evaluated more frequently until the time for the dead settled mussels to fall off can be determined, and this lag time incorporated into analysis.

Reporting

Data will be summarized and reported on a monthly basis. The optimal settlement prevention treatment will be based on when there is positive settlement on control plates but 0% settlement, or 0% settlement increase (ratio with control), on treated plates while also achieving low treatment times and doses.

Literature Cited

Claudi, R. and G. L. Mackie. 1994. Practical manual for zebra mussel monitoring and control. CRC Press, Boca Raton, Florida.

USACE (United States Army Corps of Engineers). 2002. *Dreissena bugensis* - Quagga Mussel. July 1, 2010. <http://el.erdc.usace.army.mil/zebra/zmis/zmishelp4/dreissena_bugensis_quagga_mussel_larva.htm>.

APPENDIX 9a

Methods for analysis of Pf CL145A/MOI 401 (Zequanox™) in water, as developed by MBI.

Stock concentrations of Pf CL145A/MOI 401 are determined by laboratory tests that quantify dry cell weights (DCW). These concentrations are used to calculate field application rates. However, analysis of DCW during field applications is not possible. Other analysis techniques have been developed to measure diluted product concentrations in water during applications. These include measurement of the following parameters:

- Total organic carbon (TOC)
- Turbidity, typically measured in nephlemetric turbidity units (NTU)
- Pseudomonas enumeration (live bacterial portion only)

TOC measurements can be obtained using on-line monitoring devices, HACH® test kits, and standard lab TOC analyzers. Turbidity is typically measured using on-line monitoring devices or hand held measuring devices. Since turbidity measures the light scattering properties in water, turbidity values can be obtained within a few minutes. TOC measurements typically take at least 20 minutes for quick analysis in an on-line analyzer and the HACH test kits take a number of hours.

Quantifying field levels of both TOC and turbidity is done by comparing monitoring device readings with known DCW samples. DCW samples at various known levels are used to generate response curves between the monitoring device generated data (TOC, NTU, etc.) and the actual DCW. Data from field samples can then be translated into DCW using the response curve relationship.

TOC and turbidity can be measured before and after application to determine background concentration in a water system to also determine when the product concentration is close to zero. Raw water turbidity concentrations can change rapidly, particularly during storm events. Natural TOC concentrations tend to change less drastically in natural water systems but can increase during storm events. Therefore, it may sometimes be necessary to capture raw water samples from an untreated location to determine if the product is no longer in the system. After the product has been diluted into receiving waters, TOC and turbidity are not likely accurate measurements of product concentrations due to the low contribution of TOC and turbidity into the receiving water. TOC may be viable in situations where the dilution factor is less than 100.

Enumeration of live Pseudomonas cells, not just Pf CL145A/MOI 401, can be conducted by using the attached method (APPENDIX 9b), developed by MBI. This technique is used by MBI to measure ambient Pseudomonas concentrations in the Colorado River; MBI is currently in the process of revising this method. By conducting enumeration before, during, and after treatment, any live Pseudomonas contributed by the treatment can be detected. This method only detects living Pseudomonas cells and requires 36 hours to determine concentrations. Consequently, it is best used to evaluate the treatment and assess dilution of the product into the receiving body rather than to determine if the target Pf CL145A/MOI 401 concentration has been reached in the treated system.

Marrone Bio Innovations

APPENDIX 9b

MBI Standard Operating Procedure: Enumeration of Pseudomonas sp. in water using membrane filtration method.

1. PURPOSE	The membrane filtration (MF) method is used for the presumptive identification, confirmation and enumeration of *Pseudomonas sp.* in water samples.
2. SCOPE	This protocol describes the procedure for processing water for enumeration of *Pseudomonas sp* in the field. Samples are collected, exact volume determined and filtered through a 0.45 µm membrane filter. Membrane filters are incubated on Pseudomonas isolation agar. Pseudomonas colonies are differentiated by their green pigmentation and counted.
3. MATERIALS	Pseudomonas isolation agar poured into 60mm diameter Petri plates Stainless Steel forceps Alcohol burner 70 or 90% ethanol for sterilization 500 ml filtration unit with funnel Gridded filter membranes (sterile and individually packed), 0.45µm Buffered sterile water (50 ml aliquots) Graduated cylinders (100ml and 1000ml) Sealable plastic bags Parafilm Hand-operated vacuum pump Ice or blue-ice packs Insulate shipping box or ice chest
4. METHOD	**4.1 Materials preparation** 4.1.1 Pseudomonas Isolation Agar (PIA) plates are supplied by the microbiology laboratory. Request the adequate number of plates from the microbiology lab a week in advance. 4.1.2 Label all media plates with the date and sampler initials. Discard all media with an expired shelf life (1 month from preparation date) 4.1.3 Store prepared Petri dishes upside down in a plastic bag before use and refrigerate. **4.2 Membrane filtration procedure** 4.2.1 Select a suitable work area inside the field vehicle, and out of direct sunlight and wind. 4.2.2 Select the correct sample volume to result in at least one filter having colonies in the ideal counting range. If necessary, several volumes/dilutions of the same samples should be filtered and plated. 4.2.3 Assemble filtration equipment by placing the filter –holder assembly onto the receptacle vessel. Vacuum is supplied by use of a hand-held pump.

	4.2.4 Sterilize stainless steel forceps by immersing tips in a small bottle or flask containing 70 or 90% ethanol, then pass forceps through open flame of an alcohol burner. Allow alcohol to burn out and forceps to cool for several seconds. Hot forceps will damage the membrane filter. 4.2.5 Membrane filter are individually packed and sterile. Open membrane packs one at a time as needed. Handle the membrane with clean sterile forceps only at the corners of the membrane filter. 4.2.6 Place the membrane on the filter-holder assembly. Make sure the membrane is grid-side up. 4.2.7 Place the filtration funnel on top of the filter holder assembly and screw on, making sure a tight seal forms. Avoid tearing or creasing the membrane filter. 4.2.8 Rinse funnel with 100 ml of sterile buffered water before filtering samples. This will provide the **filter blank**. Use a fresh sterile membrane filter for each sample. 4.2.8.1 Filter samples in order of smallest to largest sample volumes. 4.2.8.2 If the sample volume is between 1 and 10 ml, pour about 20 ml of sterile water of buffer into the funnel before pipetting the sample to facilitate distribution of bacteria on the membrane filter. 4.2.8.3 If the sample volume is 10 ml or more, it can be directly transferred to the funnel and filtered directly. 4.2.9 Apply vacuum with a hand-help pump. To avoid damage to the bacteria, do not exceed a pressure of about 5 lb/in^2 or 25 cc mercury. 4.2.10 Rinse inside of funnel with 20-30 ml sterile buffered water while applying vacuum. If a graduated cylinder or pipet was used to handle the sample, rinse them with sterile buffered water and deliver rinse water to the filtration apparatus. 4.2.11 Remove the funnel and hold it in one hand. Do not set funnel on the countertop. Remove the membrane with sterile forceps. Release the vacuum. Releasing the vacuum after removing the filter prevents backflow of sample water onto the filter. Unnecessarily wet filters promote confluent growth of colonies and poor results. Replace the funnel on filter base. 4.2.12 Open Petri dish and place membrane filter grid side up on medium by use of a rolling action, starting at the edge. Avoid trapping air bubbles under the membrane filter. Do not expose prepared plates to direct sunlight. 4.2.13 Close Petri dish and seal by wrapping two times around with parafilm. 4.2.14 Continue to filter the other sample volumes in order, from smallest to largest volume. Record all volumes filtered and time of processing. **4.3 Incubation** 4.3.1 Double check that all Petri dishes are properly sealed with parafilm

	and clearly labeled. 4.3.2 Pack all Petri plates in a sealable plastic bag and place on ice immediately. 4.3.3 Plated samples should be shipped back to the microbiology lab as soon as possible. 4.3.4 Incubation and quantification of Pseudomonas will be performed by microbiology laboratory at Davis, CA facilities.
5. **REFERENCE**	5.1 **National Field Manual for the Collection of Water Quality Data.** USGS. Techniques for Water Resources Investigations. Handbook 9. Chapter A7, Biological Indicators. 5.2 **Comparative study of selective media for enumeration of Pseudomonas aeruginosa from water by membrane filtration**. A de Vicente, J J Borrego, F Arrabal, and P Romero. Appl Environ Microbiol. 1986 April; 51(4): 832–840.
6. VERSION	1.0
7. APPROVAL	Prepared by _____ Date _____ Ana Lucía Córdova-Kreylos Senior Scientist, Microbiology Approved by _____ Date _____ Marja Koivunen VP of Research and Devlopment

APPENDIX 10

Product Dilution Summary

Point of Use Discharge

Point of use discharge examples include application within enclosed or semi-enclosed water systems in which the product is injected into the enclosed or semi-enclosed conduit (pipe or water conveyance channel) via a chemical injection pump. After the treated water flows through the system it is then discharged back into the open water. The most common example of this is direct surface water withdraw for cooling water systems in power facilities.

Description of Product Application

A standard chemical feed tank with an outlet connected to a chemical injection pump is filled with the desired amount of product to be applied. The pump withdraws product from the chemical feed tank and injects it into the conduit to be treated at a desired delivery rate (completely mixed concentration no greater than 200 mg/L). Standard Injection ports and mixing injection systems are used to inject the product. The equipment is similar to, if not the same as, existing equipment used in facilities currently treating for invasive mussels with proprietary biocides or chlorine. Product is supplied in a very similar manner in which chemicals are added in drinking water and wastewater treatment processes.

Overview of Dilution Calculations

Typical run-of-river hydropower facilities consist of various systems that utilize water to generate power. Of particular interest in this case is cooling water which is used to cool equipment components within hydropower facilities prior to being released back to the river via the power plant tailrace. The presence of invasive mussels in these systems inhibits the cooling processes primarily through flow restriction. Only a small portion of the total volumetric flow rate passing through a hydropower plant is used for cooling purposes. The majority is passed through the turbines. It is this untreated turbine discharge that constitutes the dominant product dilution mechanism during treatment of a cooling water system. The product dilution for cooling water system point of discharge applications at power and industrial facilities can be estimated based on the following equation and assumptions:

$$C_C Q_C = C_D(Q_C + Q_T) = C_D Q_R$$

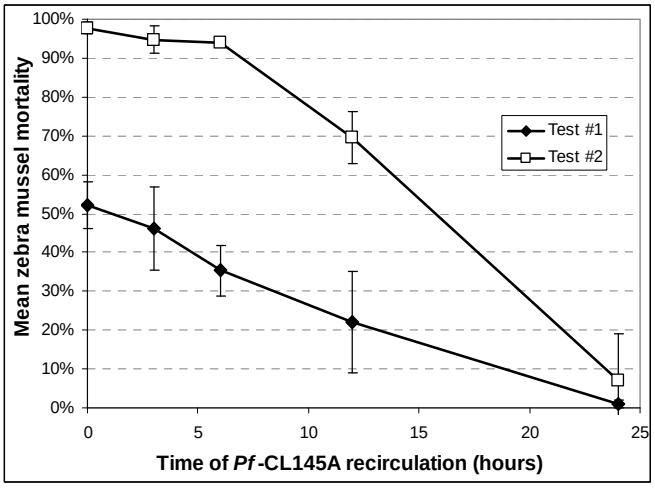
C_C = Treatment concentration in cooling water system
Q_C = Treated water volumetric flow rate (cooling water system discharge)
C_D = Diluted concentration downstream of point of discharge (power plant tailrace)
C_T = Untreated volumetric flow rate (turbines discharge)
Q_R = Total release volumetric flow rate (cooling water and turbine discharges).

For this analysis, it is assumed that no initial concentration of the product exists upstream of the power plant to be treated and complete mixing between the cooling water and turbine discharge streams will occur in the power plant tailrace which is referred to as point of discharge. This rapid mixing assumption is considered justified, particularly for hydropower facilities, because water discharged through turbines produces large magnitudes of turbulence intensity in the tailrace.

Concentrations of product downstream from the point of discharge could also be estimated using a similar equation to account for further dilution contributed by tributaries or other known raw water sources.

With respect to product decay, MOI-401 EP activity decreases in water within 24 hours, as presented in Figure 1. Hence, the product concentration in a receiving body at the point of discharge continues to decrease downstream due to decay and attenuation by organic material in the receiving body. However, these processes are dependent on individual receiving water bodies.

Figure 1. Degradation of Pf-CL145A efficacy over time. Hard water was treated at an initial concentration of 120 ppm (Test #1) and 105 ppm (Test #2) and then mussels were exposed over time.



Treatment Example: Colorado River Hydropower Facilities

As previously discussed, the majority of the water flowing through a hydropower plant is used to produce power. Cooling water and other service water systems typically use reservoir water from independent intakes separate from water released through the turbines. For most hydropower facilities, cooling water flow rates do not significantly vary day to day.

The majority of Colorado River water that is impounded by Hoover, Davis, and Parker Dams is used to produce power and hence released back to the river through the power plants. There are certain situations for which untreated water may be released through spillways or outlets other than power plants. However, for Colorado River hydropower facilities, such releases occur infrequently and are typically of short duration. Therefore, the discharges through the power plants are assumed to be representative of total release volumetric flow rates through the dams and thus the volumetric flow rates in the river.

Table 1 presents the diluted product concentrations using treated cooling water flow rates, minimum and maximum turbine flow rates, and minimum daily average turbine flow rates for Hoover, Davis, and Parker Dams. The total discharge from a hydropower facility will change (through changes in turbine discharge) during the day and year depending on power demand, water rights and in-stream flow requirements. As such, the maximum river concentrations were calculated using the minimum turbine discharges at each of the dams. The concentration at point of discharge assumes an applied product concentration (C_C) of 200 mg/L in the cooling water system which is the maximum application concentration of MOI- 401 EP. As previously noted, C_D will continue to decrease since MOI-401 EP does not persist due to decay and binding with organic matter.

Table 1. Turbine discharges, cooling water flow rates, % cooling water flow rates to turbine discharges, and maximum concentration of MOI-401 EP in river at point of discharge.

Dam	Min. turbine discharge (cfs)	Max. turbine discharge (cfs)	Min. daily avg. turbine discharge (cfs)	Total cooling water flow rate (cfs)	% treated cooling water flow rate to max. turbine discharge	% treated cooling water flow rate to min. turbine discharge	Min. POD[1] conc. (mg/L)	Max. POD conc. (mg/L)	Max. daily avg. POD conc. (mg/L)
	Q_{Tmin}	Q_{Tmax}	Q_{Tavg}	Q_C	Q_C/Q_{Tmax}	Q_C/Q_{Tmin}	C_{Dmin}	C_{Dmax}	C_{Davg}
Hoover	1330	52800	9989	47.5	0.09	3.57	0.18	7.14	0.95
Davis	4930	25000	8685	36.3	0.15	0.74	0.29	1.47	0.84
Parker	1370	20000	5932	11.0	0.06	0.80	0.11	1.61	0.37

[1] POD, point of discharge

Background *Pseudomonas spp*. Concentrations in Selected Locations on the Colorado River

Introduction

Water samples are collected at selected locations on the Colorado River to determine the ambient *Pseudomonas* spp. concentration present in the Colorado River throughout the year. Water samples are being analyzed for *Pseudomonas* spp. concentration to determine the ambient *Pseudomonas* concentrations in the environment to support regulatory approval and environmental acceptance of Zequanox, an invasive zebra and quagga mussel control product comprised of *Pseudomonas fluorescens* CL 145A strain. Zequanox^{TM} is comprised of the dead *Pseudomonas* cells, therefore, when applied there should not be detectable concentrations in water bodies that receive water treated with Zequanox.

Water samples are collected, filtered, handled, and enumerated according to the attached standard operating procedure entitled "Enumeration of Pseudomonas species in water using membrane filter method." This method is based on a method developed by United States Geological Survey for environmental heterotrophic plate counts found in the National Field Manual for the Collection of Water Quality Date. Sampling was initiated in January 2010 after extensive method validation. Sampling will continue on a monthly basis for two years at the selected locations to observe seasonal trends and develop records of natural concentrations in the environment.

The filtered water samples are incubated over *Pseudomonas* isolation agar (PIA) and the *Pseudomonas* colonies are distinguished by their fluorescence. The method enumerates the *Pseudomonas* species that grow on the PIA and does not specifically identify *Pseudomonas fluorescens*.

Sample Locations

Colorado River Sampling Locations

The Colorado River Locations samples collected above Davis Dam, from the cooling water system in Davis Dam and downstream of Davis Dam.

Colorado River, Katherine's Landing, Lake Mohave, AZ 35°12'58.10"N,-114°33'56.51"W
Davis Dam Cooling Water System AZ 35°11'45.26"N,-114°34'12.87"W
Colorado River, Davis Camp, Bullhead City, AZ 35° 11'36.35"N,-114° 34'15.41"W

Results

Pseudomonas spp. concentrations are determined based on the fluorescing colony forming units (cfu) divided by the sample volume filtered and reported in cfu/ml. Table 1 and Table 2 list the temperature, average concentration and standard deviation for the Colorado River locations.

Table . *Pseudomonas* spp. concentrations at selected Colorado River locations, above Davis Dam, from the Davis Dam cooling water system, and below Davis Dam

Colorado River, Katherine's Landing, Lake Mohave, AZ 35°12'58.10"N,-114°33'56.51"W			
Collection Date	Water Surface Temperature °C	Concentration (cfu/mL) Average	Standard Deviation
1/26/2010	-	1.38	1.26
2/25/2010	-	2.01	1.13
5/19/2010	20	0.91	0.59
6/24/2010	18	5.83	3.48
7/8/2010	20	0.61	0.65
7/27/10	25	6.67	3.71
8/17/10	27	1.20	1.30
9/28/10	20	2.56	0.98
10/19/10	20	1.83	2.28
11/16/10	16	2.00	1.11
1/3/11	10	12.6	3.08
Davis Dam Cooling Water System AZ 35°11'45.26"N,-114°34'12.87"W			
Collection Date	Water Surface Temperature °C	Concentration (cfu/mL) Average	Standard Deviation
1/26/10	-	0.34	0.41
2/25/10	-	0.93	0.70
3/25/10	-	37.28	39.39
5/19/210	20	0.25	0.34
6/24/2010	19	12.67	3.50
7/8/2010	20	10.67	6.58
7/27/10	16	3.70	3.83
9/28/10	19	2.00	2.11
10/19/10	20	4.00	2.75
11/16/10	17	2.87	1.33
1/3/11	12	6.5	3.12
Colorado River, Davis Camp, Bullhead City, AZ 35° 11'36.35"N,-114° 34'15.41"W			
Collection Date	Water Surface Temperature °C	Concentration Average (cfu/mL)	Standard Deviation
6/24/2010	19	0.76	0.83
7/8/2010	20	1.78	1.92
7/27/10	14	6.00	3.00
8/17/10	20	1.93	1.45
9/28/10	20	0.67	1.12
10/19/10	18	4.44	2.47
11/16/10	17	0.56	0.70
1/3/11	9	2.83	1

Appendix D

Zequanox™ Product Label

THIS IS AN UNREGISTERED PESTICIDE PRODUCT FOR USE ONLY BY THE U.S. DEPARTMENT OF INTERIOR BUREAU OF RECLAMATION IN COORDINATION WITH THE FIFRA SECTION 18 QUARANTINE EXEMPTION FOR THE USE OF ZEQUANOX FOR CONTROL OF QUAGGA AND ZEBRA MUSSELS IN ARIZONA, CALIFORNIA AND NEVADA

Effective Date: ..... Expiration Date:

Zequanox

(ALTERNATE NAME: MOI-401 SDP)

ACTIVE INGREDIENT:
Pseudomonas fluorescens strain CL145A cells* 50.0%
OTHER INGREDIENTS:..... 50.0%
TOTAL:..... 100.0%
*Contains no more than 10 CFU/g of *Pseudomonas fluorescens* strain CL145A cells.

KEEP OUT OF REACH OF CHILDREN

WARNING/AVISO

Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detaile.
(If you do not understand the label, find someone to explain it to you in detail.)

FIRST AID	
If inhaled	• Move person to fresh air. • If person is not breathing, call 911 or an ambulance, then give artificial respiration, preferably mouth-to-mouth if possible. • Call poison control center or doctor for treatment advice.
If in eyes	• Hold eye open and rinse slowly and gently with water for 15 – 20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
If swallowed	• Call poison control center or doctor immediately for treatment advice, • Have person sip a glass of water if able to swallow. • Do not induce vomiting unless told to do so by the poison control center or doctor. • Do not give anything by mouth to an unconscious person.
If on skin or clothing	• Take off contaminated clothing. • Rinse skin immediately with plenty of water for 15 – 20 minutes. • Call a poison control center or doctor for treatment advice.
HOT LINE NUMBER Have the product container or label with you when calling a poison control center or doctor, or going for treatment. You may also contact 1-800-222-1222 for emergency medical treatment information.	

EPA Est. No.: 84059-CA-001
Batch / Lot No.:
Net Contents:

PRECAUTIONARY STATEMENTS

HAZARDS TO HUMANS AND DOMESTIC ANIMALS – WARNING: May be fatal if inhaled. Do not breathe dust, vapor or spray mist. Wear a respirator with a dust/mist filtering canister meeting NIOSH standards of at least N-95, R-95 or P-95 to reduce pulmonary exposure while mixing or loading Zequanox. Remove and wash contaminated clothing before reuse. Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization.

DIRECTIONS FOR USE

It is a violation of federal law to use this product in a manner inconsistent with its labeling.

This label must be in the possession of the user at the time of pesticide application.

Sites to be Treated: Sites infested with invasive dreissenid mussels, specifically: a) three Lower Colorado River Dams (Hoover, Davis, and Parker and their associated power generation facilities, e.g., trashracks, cooling pipes and plumbing) where ample dilution of discharges occurs; and b) a piped-irrigation water distribution system of the Coachella Valley Irrigation District in areas where treated water is not returned to natural, fish-bearing waters. ZEQUANOX will be used to control both adult and juvenile (also known as veligers) dreissenids.

Method of Application: ZEQUANOX will be applied using standard aquatic pesticide application equipment and or similar equipment commonly used for chemical injection in drinking water treatment. This includes equipment such as sprayers, mixers, injection pumps and/or weighted hoses. The material will be contained and transported in totes or appropriate plastic chemical application barrels. Application will be flow of volume based. For enclosed and confined systems (i.e. canals, irrigation, and pipes), treated water flow rates and chemical injection pump flow rates can be measured by using flow meters and hand flow measurements. Turbidity measurements before and after application can be used as a surrogate to measure actual applied product .

Maximum Rate of Application: Up to 200 ppm a.i. for up to 24 hours per month (4 week period).

Maximum Number of Applications: After the initial submission of the Section 18, MBI demonstrated the effectiveness of ZEQUANOX in preventing the settlement of juvenile/larval veligers in addition to adult mussels. This provides an important barrier to protect Reclamation facilities within the cooling water systems that have numerous small pipes and orifices that can easily become clogged from mussel shells, even after adult mussels are dead. Infested systems will be treated at high concentrations to remove adult mussels prior to settlement maintenance treatment initiation for sustained control. Settlement maintenance techniques would be similar to chlorine treatments in that lower doses are periodically pulsed into the system over time.

ZEQUANOX will therefore be applied by one of two scenarios, depending on the system, level of infestation, stage within mussel lifecycle, time of year, sensitivity of the system to abrasion/occlusion, etc:

1.) Rehabilitation Level Treatment.

The purpose of this treatment is to kill and/or remove attached adult mussels from infested systems. These treatments would be at concentrations near or at the maximum rate of 200 ppm a.i. for a continuous 24 hour period. No more than one of these treatments will be conducted per month per site for the duration of the Section 18.

2.) Settlement Maintenance Level Treatments

Settlement maintenance is an on-going treatment during the mussel spawning season and prevents juvenile mussels from settling and growing to the adult stage within the system. It is performed in smaller pipes and orifices that are more susceptible to damage mussel settlement, and prevents shell debris clogging or abrasion damage.

In the Colorado River, a single mussel can grow to a size at which it could significantly limit flow or completely occlude smaller piping within a 6 month time period. Because mussels appear to be reproducing almost year round on the Colorado River (as opposed to about 6 months in the North East), the growth cycle may require numerous adult treatments per year or regular settlement maintenance treatments. Many facilities in the North East that are highly sensitive to shell debris, typically to protect small piping in cooling water systems, currently conduct regular settlement maintenance using chlorine to prevent facility shutdown and loss of operation.

A minimum of 10 mg/L will be applied for continuous durations of no more than 2 hours per day. Each 2-hour application will be repeated no more than 3 times per week throughout the year, for a total of no more than 24 hours of treatment per 4-week period. These types of pulsed treatments are considered as a single annual application (i.e. one per site per year).

Regardless of the treatment scenario employed, the concentration at any time will not exceed 200 ppm a.i., contiguously applied for no longer than 24 hours and not to exceed a combined (non-contiguous) total of 24 hours per 4-week period. The total annual amount of ZEQUANOX end-products used will not exceed the maximum stated below.

Maximum Amount of Pesticide to be used: 60,000 kg active ingredient per year.

Maximum Volume to be treated: Based on the maximum amount of pesticide to be used, and the minimum treatment rate of 10 mg a.i./L (Settlement Maintenance Level Treatments), the maximum volume of water that will be treated will be 4865 acre-feet. The use of the maximum treatment rate (Rehabilitation Level Treatments) at 200 mg a.i./L will reduce the total volume treated, given the maximum amount used of 60,000 kg a.i. (minimum of 243 acre feet, corrected value).

Use Season: Treatments will be made as needed year-round beginning immediately, and either until the Section 3 registration for ZEQUANOX is completed or for the duration of Section 18 approval.

Additional Restrictions, User Precautions and Requirements, Qualifications of Applicators, etc.: All personnel who work with ZEQUANOX will be certified pesticide applicators and will use and be trained in the use of required and appropriate personal protection equipment as per OSHA

standards, the product MSDS, and the product label. Working with ZEQUANOX (including handling and storage, process replenishment and housekeeping activities) requires worker to use appropriate eye and face protection, gloves, and impervious clothing.

Prior to commencing treatment process work on-site, Reclamation will prepare a job hazard analysis, which will detail emergency response and spill response measures, to include emergency phone numbers and locations of nearby emergency facilities. In addition, pre-job safety briefings will be conducted prior to each treatment.

Calculation of Application Rates:

For all applications, prior to product application, dilute Zequanox into double contained plastic injection tank, tote, or similar container appropriate for use in chemical application in aquatic environments. Add 1 kg (or 2.2 lb) of dry Zequanox to the plastic container then add 8 to 13 liters of non-chlorinated water to achieve a slurry concentration of 125 to 77 g/L of total Zequanox or 75 to 38 g/L as active ingredient. Mix well. Once Zequanox is diluted, follow application instructions as described below.

Enclosed, Semi-enclosed, and other confined flowing water infrastructure

For adult zebra and quagga mussels control in enclosed, semi-enclosed, and confined flowing water in infested infrastructures, e.g. pipes and any water conveyance structures, associated with civil infrastructure such as, power plants, industrial and manufacturing facilities (e.g. automobile and steel), dams, and irrigation systems, inject diluted Zequanox contained in the appropriate chemical injection tank (container) into flowing water with standard chemical injection metering pump to reach a completely mixed and homogeneous suspension of up to 200 mg per liter concentration of active ingredient within flowing water. Maintain continuous injection with the chemical metering pump production for 1 to 24 hours. To achieve the maximum desired concentration, calculate the injection rate (volumetric dose) based on the total volumetric water flow rate (or volume) and diluted product concentration.

For settlement prevention control of juvenile and planktonic zebra and quagga mussel life stages (veliger life stage) in enclosed, semi-enclosed, and confined flowing water in infested infrastructures, e.g. pipes and any water conveyance structures, associated with civil infrastructure such as, power plants, industrial and manufacturing facilities (e.g. automobile and steel), dams, and irrigation systems, inject diluted Zequanox contained in the appropriate chemical injection tank (container) into flowing water with standard chemical injection metering pump to reach a completely mixed and homogeneous suspension of up to 50 mg per liter concentration of active ingredient within flowing water. Maintain continuous injection with the chemical metering pump production for 1 to 6 hours. To achieve the maximum desired concentration, calculate the injection rate (volumetric dose) based on the total volumetric water flow rate (or volume) and diluted product concentration. Repeat injection up to three times a week, dependent on viable veliger density, continuously through mussel spawning season.

Open non-flowing or low-flowing water bodies

For juvenile or adult mussel control in open non- or low-flowing bodies of water, e.g. ponds, lakes, reservoirs, apply diluted Zequanox to infested areas to obtain up to 200 mg per liter concentration of active ingredient within two feet of mussel infested surfaces from appropriate chemical injection tank. Pump diluted Zequanox from the chemical injection tank through tubing

and diffusers to spread product at the surface or subsurface. Apply product at the surface by boat or appropriate hoses for product application on the shore of an open water body.

For veliger and larval stage control in open water systems, e.g. ponds, lakes, reservoirs, directly apply Zequanox to infested water column to obtain up to 50 mg per liter concentration of Zequanox active ingredient.

Specialized equipment deployed in aquatic environments

For equipment deployed in aquatic environments, pour or inject using a chemical metering pump, Zequanox into desired fully contained treatment area. Once a completely mixed and homogeneous suspension of up to 200 mg per liter of Zequanox active ingredient has been achieved, keep treated area contained for 12 to 24 hours. After desired treatment time has been achieved, remove containment to release treated water.

Product measurement in treated system

Perform turbidity measurements to determine when the desired completely mixed homogenous concentration of Zequanox active ingredients is achieved and to the required Zequanox active ingredient concentration. In order to correlate target turbidity to desired active ingredient concentration, add the necessary volume of diluted Zequanox to achieve the target concentration into a known volume of water contained in a plastic or glass container and mix. Read turbidity of this mixture. This is the target turbidity for the desired completely mixed homogenous concentration.

After application, allow 2 to 4 weeks, respectively, at warm ca. >20ºC (68°F) to cold ca. <10ºC (50°F) water temperatures before determining the final mortality achieved from each treatment.

STORAGE AND DISPOSAL

Do not contaminate water, food or feed by storage or disposal.

Pesticide Storage: Store in original container, in a cool, dry place.

Pesticide Disposal: To avoid wastes, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments of by industry).

Container Disposal: Non-refillable container. Do not reuse or refill this container. Triple rinse (or equivalent) promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank and drain for 10 seconds after the flow begins to drip. Fill the container ¼ full with water and recap. Shake for 10 seconds. Pour rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Drain for 10 seconds after the flow begins to drip. Repeat this procedure two more times. Then offer for recycling if available or puncture and dispose of in a sanitary landfill, or by incineration. Do not burn, unless allowed by state and local ordinances.

Upon expiration of the emergency use exemption, any unused, unregistered product must either be returned to the manufacturer or distributor (unopened containers) or disposed of in accordance with Resource Conservation and Recovery Act regulations.

WARRANTY

To the extent permitted by applicable law, the seller makes no warranty, expressed or implied, of merchantability, fitness or otherwise concerning use of this product. The user assumes all risks of use, storage or handling that are not in strict accordance with the accompanying directions.

Manufactured by: Marrone Bio Innovations, Inc.
2121 Second St., Suite B-107
Davis, CA 95618 USA

Appendix E

Manufacturer’s Material Safety Data Sheet

Product Name: MOI-401 SDP

Trade names/ Synonyms: Zequanox
EPA Registration Number: None, Experimental
Primary Hazards: Inhalation

Contact: Marrone Bio Innovations, 2121 Second Street,
Suite B-107, Davis, CA 95618
Phone (Business hours): 530-750-2800
www.marronebioinnovations.com
For emergencies such as leaks or spills call CHEMTREC 24-hour toll-free hotline at 1.800.424.9300

SECTION 1: MATERIAL IDENTIFICATION

INGREDIENT 1

Common Name:	CL145A strain of *Psuedomonas fluorescens* cells, powder formulation
Chemical Name:	Not applicable
Molecular Formula:	Not applicable
CAS Number:	Not applicable
Percent:	80%

OTHER INGREDIENTS: inert, non-reactive

SECTION 2: PHYSICAL DATA

Boiling Point:	Not applicable
Melting Point:	Not applicable
Density:	1.05 g/mL
Solubility in Water:	Dispersible in water
Appearance:	Powder
Color:	Tan
Odor:	Sweet, musty

SECTION 3: FIRE AND EXPLOSION DATA

Flash Point:	Not flammable
Method:	Not applicable
Extinguishing Media:	Use extinguishing media appropriate for the surrounding fire
Special Fire Fighting Procedures:	None
Unusual Fire and Explosion Hazards:	None

SECTION 4: REACTIVITY

Stability:	Material is non-reactive
Hazardous Polymerization:	Does not occur
Incompatibility:	None known
Hazardous Decomposition Products:	None known
Conditions to avoid:	None known

SECTION 5: HEALTH HAZARDS: POTENTIAL SENSITIZER

Primary Route of Entry:	Skin contact, Eye, Inhalation
Exposure Limit:	Not established
Corrosive:	Not corrosive
Inhalation:	Do not breathe dust, vapor or spray mist. Wear a respirator with a dust/mist filtering canister meeting NIOSH standards of at least N-95, R-95 or P-95 to reduce pulmonary exposure while mixing or loading Zequanox. Remove and wash contaminated clothing before reuse. Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization.
Skin/ Eye Irritation:	May be irritating to skin and eyes for some individuals.
Effects of Overexposure:	If product comes in contact with eyes or skin, irritation may occur.
Toxicity:	None of the components of this product are listed as carcinogenic by NTP, IARC, or OSHA

Acute studies:

Acute Oral LD_{50} (Rat):	>5,000 mg/kg (very low toxicity)
Acute Dermal LD_{50} (Rabbit)	>2,000 mg/kg (non-irritating, mild or slight irritation)
Primary Dermal Irritation	Slight Irritation, Class 4
Eye Irritation	Minimal Irritation, Class 4
Inhalation	>2.25 g/mL, Class 4

SECTION 6: FIRST AID

Emergency First Aid Procedures:

If in eyes:	Hold eye open and rinse slowly and gently with water for 15-20 minutes. Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. Call a poison control center of doctor for treatment advice.
If inhaled:	Move person to fresh air. If person in not breathing, call 911 or an ambulance, then give artificial respiration, preferably mouth-to-mouth if possible.
If on skin:	Take off contaminated clothing. Rinse skin immediately with plenty of water for 15-20 minutes. Call a poison control center or doctor for further treatment advice.
If swallowed:	Call a poison control center or doctor immediately for treatment advice. Have person sip a glass of water if able to swallow. Do not induce vomiting unless told to by a poison control center or doctor. Do not give anything by mouth to an unconscious person.

SECTION 7: SPILL, LEAK AND DISPOSAL PROCEDURES

Steps to be taken in case material is released or spilled:

Wear suitable protective clothing such as long-sleeved shirt, pants, waterproof gloves and shoes with socks. Carefully mop or sweep up spill and place in a closed container for disposal.

Waste disposal method: Dispose of in accordance with all applicable federal, state, and local environmental regulations.

For emergencies such as leaks or spills, call CHEMTREC 24-hour toll-free hotline at 1.800.424.9300.

SECTION 8: SPECIAL HANDLING

Respiratory:	WARNING: May be fatal if inhaled. Do not breathe dust, vapor or spray mist. Wear a respirator with a dust/mist filtering canister meeting NIOSH standards of at least N-95, R-95 or P-95 to reduce pulmonary exposure while mixing or loading Zequanox. Remove and wash contaminated clothing before reuse. Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization.
Protective gloves:	Wear gloves made of Latex or other impervious material.
Eye protection:	Safety goggles or safety glasses with side shields recommended.
Other protective clothes:	Clothing to prevent prolonged skin contact as needed such as long-sleeved shirt, long pants and shoes with socks.

SECTION 9: SPECIAL PRECAUTIONS

Precautions to be taken in handling and storing:

Do not breathe dust, vapor or spray mist. Wear a respirator with a dust/mist filtering canister meeting NIOSH standards of at least N-95, R-95 or P-95 to reduce pulmonary exposure while mixing or loading Zequanox. Remove and wash contaminated clothing before reuse. Repeated exposure to high concentrations of microbial proteins can cause allergic sensitization.

Store in a dry area inaccessible to children. Store in original containers only. Keep container closed when not in use.

Empty container completely and dispose of in accordance with all applicable federal, state, and local environmental regulations.

Wash any contamination from skin or eyes immediately. Wash hands and exposed skin before eating, drinking, smoking after work or using the toilet.

SECTION 10: SHIPPING REGULATIONS

Proper shipping name:	None
DOT Label (s) Required:	None
Freight Classification:	Insecticides, Fungicides N.O.I., Other Than Poisons. NMFC 102120 Class 60

SARA Title III Hazard Classification:

Immediate (acute) Health:	No
Delayed (chronic) Health:	No
Fire:	No
Sudden Release of Pressure:	No
Reactivity:	No

National Fire Protection Association Rating:

Health:	0
Flammability:	0
Reactivity:	0

This document set forth is based on information that Marrone Bio Innovations, Inc. (MBI) believes to be accurate. No warranty, expressed or implied, is intended. The information is provided solely for your information and consideration and MOI assumes no legal responsibility for use or reliance thereon.

Appendix F

Research and Development Project Report Summary:
Efficacy of Pf CL 145A Formulations for the control of Zebra and Quagga Mussels at DeCew II Generating Station, St. Catharine's, Ontario, Canada

RESEARCH AND DEVELOPMENT
PROJECT REPORT SUMMARY

Efficacy of *Pf* CL 145A Formulations for the control of Zebra and Quagga Mussels at DeCew II Generating Station, St. Catharines, Ontario, Canada

Prepared by:

Kelly Murray, Biofouling Project Manager
ASI Group Ltd (ASI)

and

Sarahann M. Dow, Invasive Mussel Project Manager
Keith Pitts, Vice President of Government and Regulatory Affairs
Marrone Bio Innovations (MBI)

Reviewed by:

Tony Van Oostrom
Senior Environmental Advisor, Niagara Plant Group
Ontario Power Generation

Date Prepared: April 5, 2010

ABSTRACT

Product Tested: *Pf* CL 145A Product Formulations

Location: Ontario Power Generation's (OPG) DeCew II Generating Station (DeCew), located in St. Catharines, Ontario, Canada

Testing Time Frame: May through October 2009

Permitting: Pesticide Management Regulatory Agency Research Authorization, 07-RP-08, Ministry of Environment Certificate of Approval, 9680-7PZPQU

Objective: Efficacy of *Pf* CL 145A formulations at controlling adult zebra and quagga mussels.

With increasing information on the potential impacts of inorganic biocides such as sodium hypochlorite on species in aquatic environments, Ontario Power Generation (OPG) in partnership with Marrone Bio Innovations (MBI) has performed the first Canadian treatment trials of a novel, organically derived non-chemical treatment for zebra and quagga mussel control using a specific strain of naturally occurring bacteria *Pseudomonas fluorescens* (*Pf*) (CL 145A strain). All experimental trials took place at OPG's DeCew II Generating Station in St. Catharines, Ontario to demonstrate the efficacy of the approach in an actual generating station environment and to help bring the product Pf CL 145A, to market as soon as possible in Canada. When ingested by the zebra and quagga mussels in artificially high concentrations, the mussel digestive cells, over time, hemorrhage when they come in contact with natural compounds produced by *Pf* CL 145A and the mussels die. Testing has shown that this bacterial strain's mode of action is highly targeted. Ecotoxicity testing conducted to date shows that *Pf* CL 145A is only lethal to zebra and quagga mussels and is generally non-toxic to other organisms.

Three phases of the experimental testing were undertaken under a Pest Management Regulatory Authority (PMRA) Research Authorization and a Ministry of Environment (MOE) Certificate of Approval to determine the efficacy of various product formulations. The initial static tests involved exposing adult mussels to various *Pf* CL 145A formulations in small volumes of water (jar testing) to confirm the expected efficacy of the product. The second phase of testing, pilot scale, was conducted in a small-scale flow through environment (bioboxes), where the mussels were continually exposed to the product for a set length of time. This testing was done to compare the efficacy of the product to the static jar trials and to test improved *Pf* CL 145A formulations. During this time period, MBI continued to conduct jar testing and pilot scale trials at other locations within the United States. The final phase of experimental testing involved a full facility trial where the entire service water system at DeCew II was treated with *Pf* CL 145A for mussel control. Adult mussels were placed into bioboxes that received treated cooling water to quantify mussel mortality after the treatment. A concentrated aqueous solution containing a derivative of *Pf* CL 145A was used in all phases of testing.

Biobox and jar assay results with the technical active grade ingredient consistently showed greater than 90 percent mortality.

The full facility treatment resulted in less than 10 percent adult mussel mortality due to loss of activity in the material during final processing stages of the first commercial-scale production run of *Pf* CL 145A. Observations of juvenile and pediveliger mussels two days after full facility treatment at DeCew, however, demonstrated 88 percent morality in these life stages. This juvenile and pediveliger mortality was attributed directly to the full facility treatment of *Pf* CL 145A and has raised the prospect that even low doses of *Pf* CL 145A could be used to successfully prevent mussel settlement.

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Appendices

1.0 BACKGROUND:

This report has been prepared in accordance with condition 8(3) of Certificate of Approval 9680-7PZPQU issued by the Ministry of Environment (MOE), April 22, 2009. A copy of this report will be submitted to the Ministry of Environment Niagara District Manager. The intent of this report is to provide a summary of the results of the trial, and to summarize key elements relative to requirements in the C of A.

Niagara Plant Group (NPG) of Ontario Power Generation (OPG) has carried out sodium hypochlorite treatments to control zebra and quagga mussel infestations in piping systems since the Great Lakes were first impacted by these invasive species. While a great deal of effort has been made looking for viable alternatives that have less of an impact to the environment, there was no alternative as effective as sodium hypochlorite. During that time, NPG's focus with the help of ASI Group was on reducing the impact of sodium hypochlorite, by shortening treatment times and by making treatments more effective. Over this time, usage of sodium hypochlorite was reduced by over 80%.

When the New York State Museum (NYSM) initiated the process to commercialize the bacterium, *Pseudomonas fluorescens* (*Pf*) (strain CL 145A) with MBI, for treatment of zebra and quagga mussels, NPG approached the NYSM and MBI about doing a trial in Canada. MBI is the commercial license holder for the *Pf* CL145A. This was accepted, and after consultation with the MOE and the PMRA, plans were put in place to give this green technology a trial with the thinking, that if successful, a viable alternative would be available in the Canadian market much sooner. Ultimately, having an effective treatment alternative available on the Canadian market could result in significant reductions in sodium hypochlorite discharged to the environment if it is used by other industries to treat zebra and quagga mussel infestations.

2.0 MATERIALS AND METHODS

Three phases of experimental trials were undertaken in order to test the efficacy of *Pf* CL 145A at eliminating zebra/quagga mussels from industrial service water systems. The first phase, Bench Scale Testing (Jar Testing) tested the efficacy of *Pf* CL 145A on a small scale, static trial in order to test the efficacy of *Pf* CL 145A shipped to Canada from California and to confirm the expected efficacy of the product in DeCew II service water. The second phase of testing, Pilot Scale Testing (Biobox flow through testing), involved injecting *Pf* CL 145A into a small scale flowing environment in order to confirm the efficacy observed in previous Bench Scale testing trials and allow for product modifications to be made. The final phase of testing involved injecting *Pf* CL 145A directly into the service water system at DeCew II for a full scale, facility treatment to control zebra/quagga mussels within the entire service water system at DeCew II.

2.1 Bench Scale Testing (Jar Testing) Protocols

2.1.1 Protocols for the Collection, Handling and Maintenance of Adult Mussels

To ensure the accuracy of the mussel mortality results, which determine when the *Pf* CL 145A treatments have successfully eradicated adult mussels from a raw water system, all mussels used as an indicator of effectiveness of treatment (bioassay) must be healthy. As a result, mussels were collected, handled and maintained according to the following procedures to minimize stress related mortalities prior to experimentation.

All mussels were collected from Henley Island, located on Martindale Pond, which is fed by the Twelve Mile Creek, the same receiving water for DeCew II. Mussel harvesting occurred as needed for all trials at a minimum frequency of one week prior to all tests to ensure the health of the mussels being used. Mussels

were collected by manually removing them with a scraper from rock, pilings and other debris close to the shore. Manual scraping ensured that the mussels were removed by releasing the byssal threads and not tearing them from the mussels.

Once collected, the mussels were placed in buckets with water from the collection area and transported to the ASI Group Ltd. (ASI) laboratory located in St. Catharines, Ontario for sorting and then transported to DeCew II to be acclimated to the plant service water in the continuously flowing control biobox. Bioboxes are modified aquaria made of acrylic that are plumbed to receive a slipstream of service water (Figure 1). All mussels acclimating in the control biobox were checked periodically, at minimum of once weekly, for background mortality prior to use in any experiments. Background mortality rates exceeding 5% indicated that the health of the mussels was suspect and that new mussels must be obtained. This ensures that the mussels are healthy prior to treatment start-up ensuring that mortality is only contingent upon exposure to *Pf* CL 145A not some other factor. Mortality in the control biobox for the acclimating mussels remained at 0% throughout all experiments.

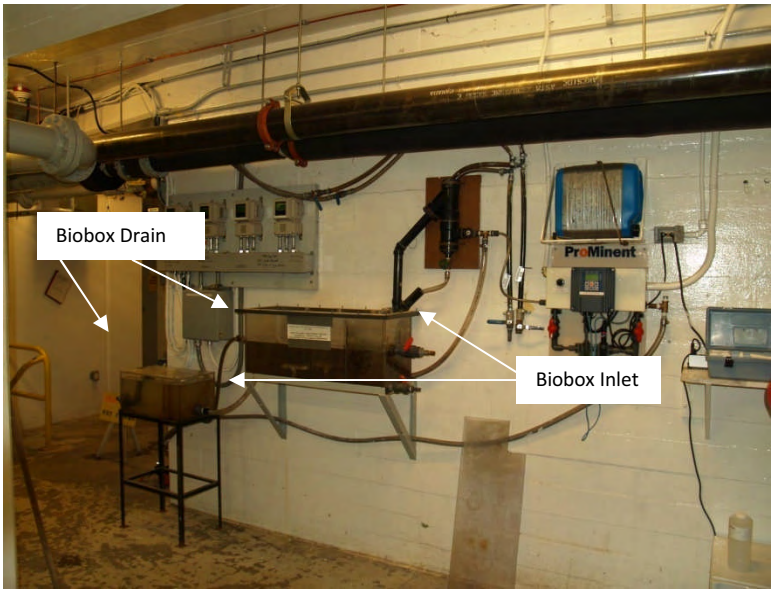

Figure 1: Testing Biobox Installation

2.1.2 Bench (Jar) Testing Protocols

Bioassays for the Bench (Jar) Scale Testing began with the seeding of the test mussels in the experimental vessels - 2 L glass pickle jars (see Figure 2) Approximately 30 adult mussels (1.0 to 3.0 cm in size), composed mainly of quagga mussels with some zebra mussels were seeded in each testing vessel with 1 L of DeCew II service water and a continuous gentle aeration 24 hours prior to injecting the vessels with *Pf* CL 145A formulations. All tests occurred in replicates of 3 with 3 control vessels which had no exposure to *Pf* CL 145A formulations. All tests and controls were seeded at the same time. After the 24 hour acclimation period, the mussels were checked for mortality and refreshed with DeCew II service water to eliminate any build up of waste material, e.g. feces that might artificially increase mussel mortality rates. After the mortality checks and refreshing, the mussels were left for a minimum of 1 hour prior to injecting with *Pf* CL 145A to ensure the mussels had resumed filtering and were not disturbed from the water change. Target concentrations of up to 200 mg/L were used in the various bench scale tests. Stock and target concentrations of *Pf* CL 145A were calculated based on a dry cell weight of the dead microorganisms and calculated using standard laboratory techniques to calculate dry weight. Water temperature in the vessels was monitored before and after refreshing each vessel with DeCew II service water.

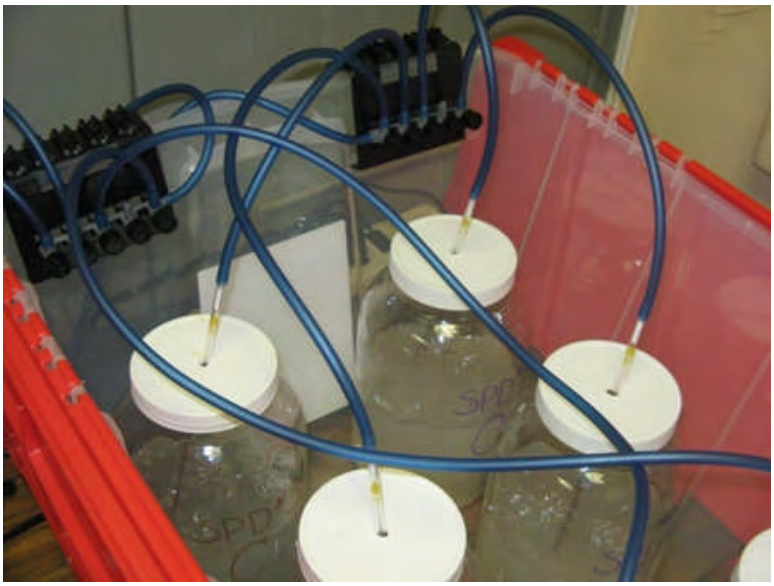

Figure 2: Bench (Jar) Scale Testing

Following the 24 hour treatment/exposure period, the mussels were checked for initial mortality. To determine if the mussels were dead, each mussel was examined individually. Per methods developed in evaluating the efficacy of chlorine treatments at facilities, mussels were considered dead if the shell was gapping open and the mussel did not respond to probing or if a closed shell mussel could easily be opened with gentle pressure along the ventral surface. Any mussels that responded to probing or could not be opened with gentle pressure were considered alive. All dead mussels were removed from the test vessel. All test and control vessels were monitored and refreshed with DeCew II service water on a daily basis for a period of up to 10 days depending on the experimental results being obtained.[1]

2.2 Pilot Scale "Biobox Flow Through" Testing Protocols

Pilot scale, flow through biobox trials were conducted at DeCew II to compare the efficacy of *Pf* CL 145A at killing invasive mussels in a flow through environment to results obtained in the preliminary bench scale tests and prior to scaling up to a full facility treatment. Zebra/quagga mussels used in all pilot scale trials were collected as described above in Protocols for the Collection, Handling and Maintenance of Adult Mussels section above. Once collected, 100 live adult mussels were separated into compartmentalized bioassay baskets which were made of plastic and were placed in the control biobox at DeCew II for acclimation to the plant service water system and to monitor mussel health prior to experimentation (Figure 3). Each basket of mussels represented one bioassay during the treatments.

In order to test the efficacy of the *Pf* CL 145A formulations at killing zebra and quagga mussels, *Pf* CL 145A formulations were injected into acrylic bioboxes, installed off of service water lines at DeCew II with continually flowing water, with the use of Manostat CARTER peristaltic pumps to inject various concentrations of the product, up to but not exceeding 200 mg/L, for a period of six hours. The *Pf* CL 145A product was injected directly into the inlet chamber of the biobox from a stock solution that was drawn from an appropriately sized container that was continually mixed with a paddle mixer to ensure the product did not settle out of solution. The product was also mixed into the receiving water in the inlet chamber of the biobox to ensure even distribution within the biobox.

[1] MBI has observed mussel mortality as far out as 60 days after one six-hour treatment with *Pf* CL 145A .

Biobox flow rates were used to help determine product injection rates and were measured by timing how long it took to fill a 2 L graduated cylinder from the biobox drain hose. Biobox flow rates were measured periodically during the six hour injection period to ensure adequate concentrations of *Pf* CL 145A product were present within the biobox and to ensure that dosing did not exceed the expected dose concentration and were not above 200 mg/L. Injection rates were modified only if significant changes in biobox flow rates were obtained.

The mussel containment baskets containing 100 live adult mussels that met all health criteria described in Protocols for the Collection, Handling and Maintenance of Adult Mussels above, were placed in the test bioboxes a minimum of 24 hours prior to beginning injection to minimize the risk of disturbing filtering behaviour during the experimental trials and were not disturbed again until the first mortality check 24 hours after product injection completion.

Pf CL 145A stock and target concentrations were calculated based on a dry cell weight of the dead microorganisms and were calculated using standard laboratory techniques to calculate dry cell weight. Target concentrations of up to 200 mg/L were selected by MBI based on previous experiments. Service water temperature was measured before and during product injection. Turbidity was monitored with the use of a Hach 2100P Turbidimeter before and during the injection to ensure target product concentrations were being obtained. In order to determine the expected turbidity, a known volume of water was dosed with a specific product volume to match the target concentration in the biobox. Biobox turbidity results throughout the six hour injection period were compared to the control turbidity results to ensure that target concentrations were being obtained in the biobox. Turbidity was also continually measured in the control biobox that was not being treated with *Pf* CL 145A to account for fluctuating turbidity in the incoming service water.

Following the six hour injection/exposure period, the mussels remained in the testing bioboxes for daily mortality checks. Service water continued to flow through the bioboxes after treatment. Mussels were checked daily for mortality in the testing bioboxes and in the control biobox. To determine if the mussels were dead, each mussel was examined individually. Mussels were considered dead if the shell was gapping open and the mussel did not respond to probing or if a closed shell mussel could easily be opened with gentle pressure along the ventral surface. Any mussels that responded to probing or could not be opened with gentle pressure were considered alive. All dead mussels were removed from the bioassay baskets.

Companion bench (Jar) scale testing was conducted during the majority of the pilot scale tests to compare the results to similar static jar assays run by MBI prior to material shipment. The companion bench scale testing was conducted to make sure there was no loss in material activity during shipment and as a control treatment to compare to the MBI jar assays with different mussels and water. Companion bench scale experiments were conducted as described in the Preliminary Bench Testing Protocol section above.

Figure 3: Bioassay Basket

2.3 Full Facility Treatment Protocols

After continued product refinement as dictated by the results obtained in the bench scale and pilot scale trials, a full scale treatment was undertaken at DeCew II on September 23, 2009. Formulated *Pf* CL 145A, electron-beamed, killed *Pseudomonas fluorescens* strain CL 145A, was placed into a clean holding tank and injected into the service water at DeCew II at two points into the main water header to an approximate concentration of 67 mg/L within the system using replacement metering pumps and the same holding tank and piping system that had been previously used for chlorination at DeCew II (Figure 4). The injection points were the same injection points as used during chlorination treatments. The need for two injection points reflects the fact that service water serves both generator 1 and generator 2 at DeCew II in a linked service water system. Two injection points provide for a more balanced injection in the service water system. Product injection using pumps with graduated calibration columns used to validate pumping rates continued uninterrupted for a period of six hours. As described in the Pilot Scale "Biobox Flow Through" Testing Protocols, turbidity samples were obtained from the testing locations (two bioboxes, with each biobox located off service water lines being fed by one of the two injection points) and compared to the turbidity control tests to ensure adequate dosing was occurring.

All treated water was directed to the tailrace of the plant after passing through the unit coolers and finally released to the Twelve Mile Creek, which included the outflow from both testing bioboxes that were plumbed to receive treated service water. The effluent in the tailrace was monitored visually prior to injection and periodically throughout the injection period to ensure that it was free of floating and settable solids and did not contain oil or any other substances in amounts sufficient to create visible film, sheen, foam or discolouration on the receiving water. Additionally, water samples were collected to determine *Pseudomonas* concentrations in the control biobox (not treated), within the service water system (treated) and in the receiving water (located downstream of the discharge from DeCew II) during the injection period to determine background levels of *Pseudomonas* species in the receiving water and to determine whether the *Pf* CL 145A treatment released in the effluent was increasing the background *Pseudomonas* concentrations in the environment downstream of the plant. All samples for *Pseudomonas* testing were shipped overnight on ice to MBI's laboratory located in Davis, California, USA for testing. Water samples were plated on King's B Agar, a

standard use agar for quantification of *Pseudomonas* species.[2] Toxicity testing results are presented in the in the "Results" section of the report.

One basket of adult mussels (Figure 3) consisting of 100 live adult zebra/quagga mussels was placed in each treated biobox as well as the control biobox a minimum of 24 hours prior to injection. Cooling system service water continued to flow through the bioboxes for the duration of the treatment exposing the mussels to *Pf* CL 145A during the entire treatment period. The mussels were left undisturbed in the bioboxes until 24 hours after the treatment was completed when the first mortality check occurred. The mussels were checked for mortality on a regular basis until it was known whether or not the treatment was successful in achieving significant mortality in the bioassays. The mortality observed from the mussels placed in the bioboxes was assumed to be the same mortality of established mussels in the service water system. Additionally, settlement plates located in one of the testing bioboxes were sampled and analyzed microscopically on September 25, two days after completing the full facility treatment, to determine if the treatment had an affect on the mortality rates of the settlement stages of larval mussels (pediveligers and juveniles) (Figures 5 & 6).

In order to directly test the effectiveness of the *Pf* CL 145A product in the service water pipes during the full facility treatment, OPG in consultation with ASI and MBI designed a pipe bypass system to act as an *in-situ* test. A "u" shaped bypass pipe was engineered and installed off a main header at DeCew (Figure 7). An access hatch was incorporated into the design to allow for in-pipe bioassay access. A stainless steel 5/32" mesh cage was designed to hold adult mussels within the pipe that could be checked for mortality on a regular basis after completion of the full facility treatment (Figures 8 & 9). To ensure that the mussels were well acclimated to the bypass; adult mussels were seeded in the mesh cage on July 6, 2009 to allow the mussels to become established within the cage (i.e. attached to the cage surfaces) mimicking established mussel populations within the service water system. The mussels were checked periodically to ensure the health of the mussels prior to the full facility treatment and were not impacted by flows, sediment build up, etc. prior to testing. The mussels were then checked for mortality after treatment completion on October 6, 2009 to determine if the treatment had an effect on these mussels. This in-situ test acted as a form of a more realistic bioassay and was included in this full scale treatment portion of the trial.

Companion bench scale, jar testing, was conducted during the full facility treatment as a product quality control to determine if any changes with the material had occurred during shipment and/or if there were any issues with product delivery during treatment. Companion bench scale experiments were conducted as described in the Preliminary Bench Testing Protocol above and at the same dose as injected into the service water lines.

[2] Extensive molecular techniques would be required in order to determine the specific *Pseudomonas* species in the environmental samples and to identify the specific *Pf* CL 145A strain. During the time of these trials, method development for environmental samples was still underway at MBI. Due to shipping the water samples, rather than filtering and plating at the time of collection there may be greater variability in the results; actual *Pseudomonas* concentrations could be either greater due to growth during shipment or less due to microbes dying in transit. Since September 2009, MBI has developed a field *Pseudomonas* sampling technique that provides more accurate and reliable results and will be used in the future to track *Pseudomonads* concentrations before and after *Pf* CL 145A treatments.

Figure 4: Existing Holding Tank & Injection System, Control Biobox in Foreground

Figure 5: Biobox Settlement Plates

Figure 6: Sampling Microscopic Settled Larvae

Figure 7: Bypass System

Figure 8: Stainless Steel Bioassay Cage from Bypass System

Figure 9: Stainless Steel Bioassay Cage in the Bypass

2.4 Toxicity Testing Protocols

Toxicity testing for non-target species was undertaken during the full facility treatment on Sept 23, 2009 to further evaluate the target specificity of *Pf* CL 145A and the environmental risk due to exposure of water streams to *Pf* CL 145A when released into the environment during mussel control treatments. Three 20 L samples of treated water were obtained from the service water system at one of the testing biobox's drain hose, additionally; one 20 L sample was obtained at the control biobox that was not exposed to *Pf* CL 145A during the treatment. The samples obtained at the testing biobox represented the treated water, while the sample obtained at the control biobox represented a site control. A lab control using ASI dechlorinated tap water was run for comparison purposes. One final sample using inert clay (kaolin clay) at a concentration of 75 mg/L was tested to determine if any mortality observed in testing could be attributed to turbidity as opposed to the product. To obtain the inert clay sample, 1.5 g of kaolin clay was added to 20 L of lab control, dechlorinated tap water with constant mixing using a paddle mixer until complete mixing was observed.

Two laboratory organisms were tested for toxicity. Forty-eight hour static *Daphnia magna* single concentration testing was performed on each of the treated water samples, the site control, the lab control and the inert clay samples. The test protocol used for all *Daphnia* testing was a standard protocol detailed in the Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*, Second Edition, Environment Canada, December 2000.

Additionally, 98 hour static Rainbow trout single concentration tests were used to determine the toxicity of all samples to Rainbow trout (*Oncorhynchus mykiss*). The standard protocol for Rainbow trout acute testing was used and is detailed in the Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout, Second Edition, Environment Canada, December 2000 and amended in May 2007.

Toxicity testing results are included in the "Results" section of the report, with supporting data in Appendix II.

3.0 RESULTS

Results below are presented under the respective type of treatment Bench (Jar), Pilot (Biobox) and Full Scale. Some results were collected that spanned treatment modes. These are presented in the Multi-mode treatment section.

3.1 Multi-Mode Treatment Results

3.1.1 Total Suspended Solids and Turbidity Monitoring Results

While Total Suspended Solids (TSS) samples were collected within 14 days of the issue date of the C of A, and then collected weekly till completion of the final full scale treatment (as per condition 7 of the MOE C of A), there was no kaolin clay used in actual treatments. Sampling was carried out because there was no option in the C of A not to sample if there was no use of kaolin clay. A decision was made early in the trial that advantages from the use of clay may not as beneficial as originally thought. Samples for TSS were sent to Exova Accutest for analysis. Turbidity samples were also collected at the same time as collection of TSS samples. Turbidity samples were obtained using a Hach 2100P Turbidimeter. Results are included in Appendix IV.

3.1.2 Bench Scale (Jar Assay) and Pilot (Biobox) Results

Independent Jar assays were conducted at DeCew II on;
- May 13, 2009 - 4 tests
- June 16, 2009 – tests
- July 24, 2009 – 2 tests

Biobox trials were conducted at DeCew II on;
- June 17, 2009 – 1 biobox trial
- July 15, 2009 – 2 biobox trials
- August 5, 2009 – 2 biobox trials
- August 18, 2009 – 2 biobox trials
- August 20, 2009 – 3 biobox trials

All biobox and jar assays were conducted per the requirements included in the PMRA Research Authorization and the MOE C of A. Note that the listed treatment date starts on the date in which mussels were seeded into tanks 24 hours in advance of product injection.

Table 1 includes the results from biobox and jar assay trials conducted with *Pf* CL 145A formulations of killed *Pseudomonas fluorescens* strain CL 145A. Initial trials in June demonstrated 55 percent mortality at a dose of 50 mg/L, which was lower than anticipated when compared with other biobox trials conducted with the same material in the United States. However, the water temperatures were colder and mussels were likely less active. In addition, at the colder temperatures when mussels are less active it can take up to 4 weeks to see greater than 90 percent mortality; mortality was only evaluated for 2 weeks on these trials. Material tested August 18 and 20, 2009 was produced at a contracted manufacturing facility in larger (10,000L) fermentation batches and processed to evaluate our ability to produce the material at a manufacturing facility. After achieving greater than 90 percent mortality on two manufacturing runs, this material and the manufacturing methods were scaled up to produce sufficient material to allow the full facility treatment to be carried out.

Also included in Table 1 are biobox and jar assay results for material that was heat shocked after fermentation to kill the *Pseudomonas* cells. This data was included to demonstrate the importance of conducting these test trials at DeCew II. This material was tested on numerous occasions in jar assays at MBI's laboratory in Davis, CA and continually demonstrated effective kill; however, when tested in biobox trials minimal mussel mortality was observed. High mortality in jar assay was also observed at DeCew II.

Table 1 Biobox and Jar Assay Trials

Treatment Date[1]	Material Description	Dose (mg/L)	Biobox % mortality	Jar Assay % Mortality (avg.)	Jar Assay % Mortality (std dev)
Concentrated material killed with e-beaming					
6/17/2009	Bench Scale Prototype	50.0	55.0		
8/18/2009	Pilot Manufactured Material	75.0	91.0	23.3	8.5
8/20/2009	Pilot Manufactured Material	75.0	96.0	91.0	6.1
Heat shocked material					
8/20/2009	Pilot Scale Material	25.0	1.0	37.7	5.0
8/20/2009	Pilot Scale Material	75.0	1.0	84.7	15.0

[1]Treatment date starts when mussels were seeded into tanks 24 hours in advance of product injection

During the bench (jar) pilot scale aspects of the trial, effluent was monitored visually prior to injection and periodically throughout the injection period of biobox flow through tests. Effluent from the jar trials was also released to the service water discharge and ultimately to the environment. At no time were free-floating and settable solids observed, nor were any oil films, sheens, foams or discolorations in the receiving waters observed (condition 5 – Visual Observations of MOE C of A).

3.2 Full Facility Treatment Results

3.2.1 Effluent Monitoring Results

During the experimental period, one full facility treatment was conducted on September 23, 2009. The full facility trial ran for 6 hours as per condition 1.1(2) in the MOE C of A. After further assessment on the use of kaolin clay, it was not used in the trial aside from being tested as part of toxicity testing discussed below. Results of TSS and turbidity monitoring were covered above.

The minimum station turbine water flow in condition 1.1(2(a) of the MOE C of A was met through the full scale trial as indicated in Table 2. Actual concentration of the *Pf* CL 145A was approximately 68 to 69 mg/l range (compared to a 67 mg/l calculated target based on volume) meeting condition 1.1(4) of the MOE C of A max concentration of 200 mg/l. The calculated effluent concentrations after mixing with turbine flow were calculated to be 0.04 mg/l (Table 2), which is well below the objective of 0.15 mg/l identified in 4(1) of the MOE C of A. Flow rates and *Pf* CL 145A concentrations are presented near the start of the treatment, in the middle and at the end as per condition 4(1) of the MOE C of A in Table 2. A description of the dilution calculations and additional calculations are provided in Appendix III.

Table 2 Effluent Objectives Results Full Facility Trial

Time		Service Water Flow (m^3/s)	Applied Pf CL145A Concentration (mg/L)	Turbine Flow (m^3/s)	Calculated Tailrace Concentration (mg/L)	Total Station Flow (m^3/s)
Beginning	11:00 AM	432	68.56	694,368	0.043	694,800
Middle	2:00 PM	429	68.96	690,771	0.042	691,200
End	5:00 PM	427	69.31	694,373	0.042	694,800

The effluent was monitored visually prior to injection and periodically throughout the injection period during the full scale trial. At no time were free-floating and settable solids observed, nor were any oil films, sheens, foams or discoloration to the receiving waters observed (condition 5 – Visual Observations of the MOE C of A).

3.2.2 Full Scale Treatment Results

An average of 14 percent mortality was observed in the companion jar assays conducted with the same material used to carry out the full scale treatment at DeCew II and less than 10% mortality was observed in the mussels in the test bioboxes that received a slip stream of treated service water. This result was less than anticipated, but related to manufacturing issues discussed below.

3.2.3 System Bypass Adult Mussel Mortality Results

As indicated above, a system bypass pipe was constructed to test the effectiveness of a full scale treatment in a real pipe environment. No mortality of adult mussels was observed in the in-pipe mussel containment that was checked on October 6, 2009 or 13 days after the full facility treatment was conducted. This was expected based on results achieved with the bioassay mussels in the biobox.

3.2.4 Identified Manufacturing Issues

MBI collected samples from the contracted manufacturing facility after each processing step in order to verify activity of the material in the MBI lab. A significant amount of testing using similar jar assay procedures was conducted at the MBI labs using these materials. After the 60,000L fermentation, the full scale production run to conduct the facility treatment at DeCew II, the material resulted in 100 percent mortality in the jar assays conducted at MBI. The material was then processed at the contracted manufacturing facility with pilot scale equipment that was not appropriately sized to the fermentation batch size resulting in excessive additional processing time and potential points of material degradation. A volume loss of about 50 percent of the material was observed after all the processing, allowing dosing at a target of 67 mg/L, rather than the desired 100 mg/L. Final samples of material after shipment sent to MBI demonstrated significant loss in activity as well. Jar assays conducted at MBI's lab dosed with 200 mg/L did show higher average mussel mortality, 80%, compared to the jar assay conducted at DeCew II dosed at 67 mg/L resulting in 14 % average mortality.. In summary, downstream processing material after fermentation was not successful.

Material produced and processed from smaller fermentation batches, pilot commercial scale runs, sufficient to supply material for multiple biobox trials demonstrated excellent mussel mortality, greater than 90 percent in trials conducted in Davis, CA at MBI's laboratories and August 20, 2009 at DeCew II, both jar and biobox trials.

MBI is currently working on these processing issues for commercial-scale manufacturing. Many of the issues identified during initial commercial runs in 2009were not apparent in earlier small-scale production batches. In addition, a great deal of information has been gained about the product performance in the field that also influences future manufacturing.

4.0 OTHER RESULTS

4.1 Data Recording and Reporting

There were no spills or loss or unplanned loss of product to the environment during the full length of the trial relative to condition 8 of the MOE C of A.

4.2 Operation and Maintenance

An Operations Manual was prepared and on site for the treatment as per Condition 2 of the MOE C of A.

4.3 Potential for Replacement of Chlorination for Zebra and Quagga Mussel Control (Condition 8(3) of the MOE C of A)

At this time, additional testing is necessary before it is possible to assess the potential for *Pf* CL 145A to replace use of sodium hypochlorite. OPG plans to request an amendment to the existing Certificate of Approval to extend the trial in 2010.

4.4 Shipments of Product from the United States

Initial plans were to have some of the product used in the trial manufactured in Canada. However, due to limited fermentation capacity at the Alberta Research Council (ARC), the identified Canadian manufacturing facility, all product used for the full scale trial ended up being produced and shipped from the United States. Some of the materials used in the biobox trials were produced by ARC but were shipped to MBI for initial testing prior to being shipped to ASI for the trials. As per condition 1(1)(9) in the MOE C of A, copies of documents authorizing the import were provided to the Niagara District Manager of the MOE as summarized in Table 3 below. Copies of these notifications are provided in Appendix V.

On July 9, 2009 the MOE District Manager was contacted regarding a clarification concern relative to a restriction on the number of test sets (Pilot). This request was granted pursuant to Condition 1.1(3) of the MOE C of A eliminating a restriction on the number of tests for the Pilot Scale (Biobox) testing. Copies of the request and response letter are included in Appendix V, item 4.

The District Manager of the MOE was notified regarding the full scale trial at least 6 days in advance as per condition 1(1)(8) of the MOE C of A and included as item 11 in Appendix V. Note this notification happened twice due to a false start on the original intended treatment date.

Table 3 Material receipt and trial notification dates and treatment dates and treatment dates for all jar, biobox, and full scale trails completed under the C of A.

	Date Notification/Request Provided	Shipping Documents Description	Treatment Date[1]
1	May 12, 2009	Jar Trial #1	May 13, 2009
	June 10, 2009	Jar Trial #2	June 16, 2009
2	June 22, 2009	Jar Trial #(3)	June 24, 2009
3	June 25, 2009	Biobox Trial #1	June 17, 2009
4	July 9, 2009	Request to Niagara District MOE for Adjustment to C of A. Reply from Rich Vickers July 9, 2009 granting additional Pilot Scale test sets as per Condition 1(1)3 of the C of A.	n/a
5	July 14, 2009	Biobox Trial #2	July 15, 2009
6	July 16, 2009	Jar Trial #4)	July 18, 2009
7	August 5, 2009	Biobox Trial #3 and #4 and Jar Trial #5	August 5, 2009
8	August 14, 2009	Jar Trial #6	August 18, 2009
9	August 18, 2009	Biobox Trial #5 and Companion Jar Trial #6	August 18, 2009
10	August 20, 2009	Biobox Trial # 6 & Companion Jar Trial # 6	August 20, 2009
11	August 26, 2009 September 8, 2009 September 17, 2009	Notification Regarding Full Scale Trial – 6 days in advance as per condition 1(1)(8) of the MOE C of A	n/a
12	September 22, 2009	Shipping Documents – Full Scale Trial	September 23, 2009

[1]Treatment date starts when mussels were seeded into tanks 24 hours in advance of product injection

5.0 BEYOND CERTIFICATE OF APPROVAL REQUIREMENTS

5.1 Larval Settlement Stage Results

As part of OPG's mussel monitoring program, settlement stage larvae (pediveligers, juveniles and adults) were monitored on a biweekly basis from June to November at the biobox at DeCew II. Existing PVC culture plates and racks in the biobox were used to track settlement densities. The plates were aligned parallel to the water flow through the biobox to provide equal exposure to water flow and reduced sampling bias to provide a more accurate assessment of settlement rates within the biobox (Figure 5). During each sampling visit plates were sampled by scraping both sides of the plate into a clearly labelled jar (Figure 6). The samples were then transported to the ASI laboratory in an insulated cooler with ice packs for microscopic analysis by experienced technicians.

Larval settlement densities were evaluated in the DeCew II testing biobox on September 25, two days after the full facility treatment program. A very high density of larval stage mussels had settled on the plates, 2 181/m^2. Of those settled on the plates 88% of were dead based on the technician's microscopic observations. Raw data on the larval settlement densities for the entire 2009 season is included in Appendix I: Settlement Densities at the DeCew NF23 Biobox Location – 2009. The high mortality rate of settlement stage larvae can likely be attributed to the full facility treatment that took place on September 23, 2009 since average mortality rates observed throughout the year remained below 6%, with one exception. During the November 6 sampling visit, average larval settlement mortality averaged 18%. This higher than normal mortality rate but can be directly attributed to the fixed interval chlorination program that took place from October 22 to November 16, 2009.

5.2 Toxicity Testing Results

Results from all 48 hour Static *Daphnia magna* single concentration tests and 98 hour static Rainbow trout single concentration tests revealed 0% mortality of testing organisms in all samples. This indicates that neither the *Pf* CL 145A formulation nor the inert clay was toxic to the non target species *Daphnia magna* or Rainbow trout. Raw data have been provided in Appendix II, Toxicity Testing Results.

Quality assurance information further supports the results from the toxicity testing. The reference toxicant results show that test reproducibility and organism sensitivity in all tests were within acceptable limits. All data was scrutinized for errors daily during the test, at test termination and during the report technical and final review stages. Instruments used to monitor parameters were calibrated daily and continuously maintained. No deviations from the protocol or operating procedure were encountered during testing. All results from the testing relate only to the sample collected and used for testing.

5.3 Measurement *of* ambient *Pseudomonas* species concentrations in Twelve Mile Creek at DeCew II

50 ml samples in plastic centrifuge tubes were shipped from DeCew II during the trials. Upon arrival, the samples were plated directly onto King's B agar to test for the presence of *Pseudomonas* species. Colonies were counted after 48 hours incubation at 25°C. Samples were plated by directly spreading 50 µl or 100 µl onto the agar using a sterile cell spreader.

Samples were collected from the cooling water system prior to treatment, during treatment, and downstream of DeCew at the Glendale Road bridge during treatment. The concentration in water was approximated using the plated volume that yielded the most appropriate number of colonies (Table 4).

These results indicate an abundant population of *Pseudomonas species* in the DeCew service water prior to treatment and no significant increase in concentration downstream of the plant, during treatment.

Table 4 Background *Pseudomonas* species Concentrations at DeCew II

Plate No.	**Sample Identification**	**Total Colonies**		**Approx. Conc. (cfu/L)**
		50 µl	100 µl	
1	Downstream from DeCew at Glendale[1]	14	95	9.5×10^5
2	DeCew Treated Water	180	TNTC[2]	3.6×10^6
3	DeCew Treated Water	TNTC	TNTC	
4	DeCew untreated water[3]	5	16	2.0×10^5
5	DeCew untreated water	12	24	18×10^5

[1] Approx. concentration calculated from 100 µl sample, 50 µl was to few

[2] To many to count, TNTC – plates had too many colonies to provide an accurate count

[3] Approximate concentrations calculated from the average of both 50 and 100 µl samples