DENTONS

Jeffrey C. Fort
Partner

jeffrey.fort@dentons.com
D +1 312 876 2380

Dentons US LLP
233 South Wacker Drive
Suite 7800
Chicago, IL 60606-6404 USA

T +1 312 876 8000
F +1 312 876 7934

Salans FMC SNR Denton
dentons.com

January 22, 2014

Via Electronic Filing and U.S. Mail

Marie Tipsord, Hearing Officer
Illinois Pollution Control Board
100 W. Randolph Street, Suite 11-500
Chicago, IL 60601

Re: R8-9 (Docket D) (Rulemaking – Water)
Follow-up submission to the December 17, 2013 Hearing

Dear Ms. Tipsord:

At the December 17, 2013 hearing the Board requested certain follow-up information to the testimony provided by Roger Klocek, on behalf of the Citgo Lemont Refinery, regarding INHS Ceriodaphnia data.

We are submitting the enclosed documents as a public comment and ask for this information to be considered by the Board. We are sending copies of this public comment to the other parties who have either provided testimony or presented questions at this hearing.

Please let me know if you have any questions or comments.

Very truly yours,

Dentons US LLP

Jeffrey C. Fort

Enclosure

cc (w/enc. via email): Stefanie Diers
Albert Ettinger
Kathy Hodge
Tom Dimond

Huff & Huff incorporated
environmental engineers
and consultants

915 Harger Road, Suite 330
Oak Brook, IL 60523
Phone (630) 684-9100
Fax (630) 684-9120
Website: http://huffnhuff.com

January 22, 2014

Ms. Marie E. Tipsord
Hearing Officer
Illinois Pollution Control Board
James R. Thompson Center
100 W. Randolph, Suite 11-500
Chicago, IL 60601-3218

Subject: R08-09 Subdocket D

Dear Ms. Tipsord,

At the Illinois Pollution Control Board's December 17, 2013, hearing (Water Quality Standards and Effluent Limitations for the Chicago Area Waterways System (CAWS), R08-09 Subdocket D), I presented information about the plankton collected in the Chicago Sanitary & Ship Canal (CSSC), at Western Avenue in Chicago. Aside from the plankton data collected by Huff & Huff, Inc., the Board requested that I provide for the record the plankton information that, at the time, was presented in the form of unpublished data collected by Dr. Steve Butler of the Illinois Natural History Survey (INHS), Champaign Urbana, Illinois. Dr. Butler collected plankton at various points on the Illinois River and the CAWS from 2010 through 2012, from May through October. This sampling effort included one station located in the CSSC at Western Avenue. Dr. Butler reported his results as *density of organisms per liter of water*. Notably, the next sample point was located downstream of the Lockport Lock and Dam where the CSSC is already in confluence with the Des Plaines River. I did not present this downstream sample location because it does not represent CSSC water exclusively. Copies of the three tables that I read from at the hearing are attached here. [1]

Specifically, I discussed the few and scattered findings of *Ceriodaphnia* from 2010-2012, from the INHS data. It is my opinion that the *Ceriodaphnia* appear to be transitory individuals that are originating in Lake Michigan and are carried along portions of the CAWS. All Cladocera (water flea) types combined, which includes *Ceriodaphnia,* appear in the highest numbers in CAWS at Lake Calumet, which is Dr. Butler's first sample site. The water flea numbers decline between Lake Calumet and the Illinois River, then increase near Ottawa Illinois, and continue to increase near Peoria. The *Ceriodaphnia* in the CSSC are not likely reproductive. Rather, *Bosmina*

[1] In addition to the INHS data, the Board also requested the Illinois River Fingernail Clam Toxicity Study conduced by Dr. Sparks. That study is included here as well.

longirostris is the dominant water flea in the CSSC, and is present in all sample months of the year. As an example, during 2012 *Bosmina* were found every month from May through October while *Ceriodaphnia* was found only in July of 2012. *Bosmina* were found at concentrations approximately 300 times greater than *Ceriodaphnia* in July 2012. *Bosmina* are a reproductive population in the CSSC.

Once water temperatures reach between 50 to 55 degrees F, photosynthesis declines dramatically and the plankton food source (single celled algae, phytoplankton) disappears from water bodies. The plankton, such as water fleas, that rely on the single-celled algae as food also disappear from these waterways at the same time.

Cladocerans, including *Ceriodaphnia,* produce "resting" (diapausing) eggs that are thickly shelled and resistant to complete drying, cold, heat and other extremes of conditions. The resting eggs are microscopic, rest in the sediment, and are often viable for years, (Kaya and Erdogan, 2013). In this protected egg state, water fleas are able to pass the winter and hatch when conditions are more favorable. Elevated chlorides in the CSSC at levels we have historically seen during winter months would likely have no effect on the hatchability of the resting eggs. (Bailey et al., 2004). Eggs will hatch when the waters warm to approximately 55 degree Fahrenheit.

In conclusion, I submit that the CSSC is not a good habitat for *Ceriodaphnia,* as evidenced by its general lack of presence or very low presence at non-continuous times during the summer months when chlorides are low. Therefore, a proposed elevated winter chloride standard would be protective of the aquatic life in the CSSC and it is my opinion that the winter chloride criterion I proposed would have no effect on the existing aquatic life that are present.

Cordially,

Roger Klocek
Senior Biologist
Huff & Huff, Inc.
915 Harger Rd., Ste 330
Oakbrook, IL 60544

References.

Bailey, Sarah A., Ian C. Duggan, Colin D.A. Van Overdjik, Thomas H. Johengen, David F. Reid, and Hugh J. Macisaac. 2004. Salinity tolerance of diapausing eggs of freshwater zooplankton. Freshwater Biology, V49, 286-295.

Butler, Steve. 2013. Personal communication. Unpublished plankton data and figures.

Kaya, Murat and Sevil Erdogan. 2013. Turkish Journal of Zoology. Morphological examination of the resting egg structure of 3 cladoceran species [*Ceriodaphnia quadrangula* (O. F. Müller, 1785), *Daphnia longispina* (O. F. Müller, 1776), and *D. magna* Straus, 1820].

NEW DOCUMENT

2010 PLANKTON COLLECTION AS MEAN DENSITY OF ORGANISMS PER LITER
AT WESTERN AVENUE, CHICAGO ILLINOIS CHICAGO SANITARY AND SHIP CANAL
ILLINOIS NATURAL HISTORY SURVEY, UNPUBLISHED DATA, STEVE BUTLER

Taxa	June	July	August	October
Rotifers				
Brachionus	18.19	18.19	16.88	6.06
Keratella	12.46	12.46	10.92	1.60
Polyarthra	52.33	52.33	52.76	18.07
Synchaeta	13.58	13.58	4.57	0.85
Trichocerca	17.84	17.84	2.22	0.60
Other Rotifers	25.31	25.31	3.97	3.50
Cladocera				
Bosminidae	0.67	0.67	4.99	1.70
Bythotrephes	0.00	0.00	0.00	0.00
Ceriodaphnia	0.00	0.00	0.02	0.00
Chydoridae	0.01	0.01	0.04	0.03
Daphnia lumholtzi	0.00	0.00	0.02	0.00
Other Daphnia spp.	0.00	0.00	0.10	0.01
Leptadora	0.00	0.00	0.00	0.00
Scapholeberis	0.04	0.04	0.00	0.00
Sididae	0.01	0.01	0.00	0.00
Simocephalus	0.00	0.00	0.00	0.00
Other Cladocera	0.06	0.06	0.14	0.00
Copepoda				
Calanoida	0.51	0.51	0.19	0.16
Cyclopoida	0.35	0.35	0.42	0.44
Harpacticoida	0.00	0.00	0.00	0.00
Nauplii	1.69	1.69	4.31	4.89
Other Taxa				
Ostracoda	0.00	0.00	0.00	0.00
Chaoborus	0.00	0.00	0.00	0.00
Dreissenidae veligers	2.04	2.04	1.81	4.33

Source: Unpublished Data, Illinois Natural History Survey, Steven Butler , no further use without Source permission

2011 PLANKTON COLLECTION WITH DENSITY OF ORGANISMS PER LITER
AT WESTERN AVENUE, CHICAGO ILLINOIS CHICAGO SANITARY AND SHIP CANAL
ILLINOIS NATURAL HISTORY SURVEY UNPUBLISHED DATA, STEVE BUTLER

Taxa	June	June	July	August	Sept
Rotifers					
Brachionus	2.15	4.71	26.88	19.00	13.15
Keratella	32.35	28.25	44.13	47.53	4.40
Polyarthra	7.30	10.11	2.90	8.59	12.55
Synchaeta	0.80	1.49	0.98	0.76	0.20
Trichocerca	0.30	1.39	6.65	7.15	5.60
Other Rotifers	12.40	13.52	32.13	38.66	5.13
Cladocera					
Bosminidae	0.07	3.39	1.99	5.78	1.22
Bythotrephes	0.00	0.00	0.00	0.00	0.00
Ceriodaphnia	0.01	0.00	0.47	0.12	0.01
Chydoridae	0.09	0.29	0.07	0.21	0.07
Daphnia lumholtzi	0.00	0.00	0.00	0.11	0.01
Other Daphnia spp.	0.03	0.12	1.03	0.03	0.01
Leptadora	0.00	0.00	0.00	0.02	0.00
Scapholeberis	0.00	0.00	0.00	0.01	0.00
Sididae	0.00	0.00	0.27	0.03	0.03
Simocephalus	0.00	0.00	0.00	0.02	0.00
Other Cladocera	0.00	0.20	0.43	0.39	0.05
Copepoda					
Calanoida	0.00	0.28	0.97	0.40	0.72
Cyclopoida	0.28	0.43	1.49	0.78	0.62
Harpacticoida	0.00	0.00	0.00	0.00	0.00
Nauplii	0.81	5.70	3.76	6.94	5.44
Other Taxa					
Ostracoda	0.00	0.00	0.00	0.01	0.00
Hydracarina	0.00	0.00	0.00	0.00	0.00
Chaoborus	0.00	0.00	0.00	0.00	0.00
Dreissenidae veligers	0.00	3.39	1.00	0.92	50.00

Source: Unpublished Data, Illinois Natural History Survey, Steven Butler , no further use without Source permission

2012 PLANKTON COLLECTION WITH DENSITY OF ORGANISMS PER LITER
AT WESTERN AVENUE, CHICAGO ILLINOIS CHICAGO SANITARY AND SHIP CANAL
ILLINOIS NATURAL HISTORY SURVEY, UNPUBLISHED DATA, STEVE BUTLER

Taxa	May	June	July	August	September	October	Lockport L&D	Lockport L&D	Lockport L&D	Lockport L&D	Lockport L&D	Lockport L&D
Rotifers												
Brachionus	20.65	9.50	15.88	88.01	4.78	2.94	3.08	100.02	64.60	238.25	21.16	1.15
Cladocera												
Bosminidae	3.26	11.12	32.87	0.76	2.86	2.11	7.23	33.52	0.28	1.95	4.31	0.64
Ceriodaphnia	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.17	0.01	0.10	0.03	0.00
Chydoridae	0.29	0.09	0.14	0.01	0.03	0.02	0.35	0.24	0.00	0.12	0.14	0.03
Daphnia lumholtzi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
Other Daphnia spp.	0.03	0.20	0.03	0.01	0.00	0.00	0.26	2.37	0.00	0.00	0.00	0.01
Leptadora	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.06	0.00	0.00
Scapholeberis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sididae	0.02	0.21	0.24	0.00	0.01	0.01	0.00	0.61	1.07	3.12	0.17	0.00
Simocephalus	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00
Other Cladocera	0.63	0.88	0.39	0.20	0.07	0.00	0.11	26.59	0.37	0.04	0.17	0.01
Copepoda												
Calanoida	0.05	0.72	2.61	0.13	0.58	0.15	0.31	1.30	0.01	0.25	0.14	0.11
Cyclopoida	0.94	2.55	2.12	0.72	0.30	0.08	0.70	4.57	0.95	1.70	0.92	0.06
Harpacticoida	0.00	11.67	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
Nauplii	4.65	5.86	10.17	2.17	2.80	1.42	2.94	6.22	2.83	2.56	1.83	1.17
Other Taxa												
Ostracoda	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.06	0.03	0.03	0.01
Hydracarina	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00
Chaoborus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dreissenidae veligers	0.00	1.63	5.17	0.19	117.00	0.97	0.33	0.00	0.00	3.33	3.42	0.56

NEW DOCUMENT

ILLINOIS

UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN

PRODUCTION NOTE

University of Illinois at
Urbana-Champaign Library
Large-scale Digitization Project, 2007.

Illinois Natural History Survey
Center for Aquatic Ecology
Technical Report 93/5

F-94-R

ILLINOIS RIVER FINGERNAIL CLAM TOXICITY STUDY

July 1, 1990 - December 31, 1991

Final Report to

Illinois Department of Conservation

by

Richard E. Sparks and Frank S. Dillon

Illinois Natural History Survey
River Research Laboratory
Forbes Biological Station
P.O. Box 590
Havana, IL 62644

Richard E. Sparks
Richard E. Sparks
Principal Investigator

Robert A. Herendeen
David P. Philipp, Director
Center for Aquatic Ecology

Robert A. Herendeen
Acting Director

DISCLAIMER

The findings, conclusions, and views expressed herein are those of the researchers and should not be considered as the official position of either the U.S. Fish and Wildlife Service or the Illinois Department of Conservation.

Mention of product names and vendors does not imply endorsement by the U.S. Fish and Wildlife Service, the Illinois Department of Conservation, or the Illinois Natural History Survey.

ACKNOWLEDGEMENT OF SUPPORT

The Illinois River Fingernail Clam Toxicity Study (F-94-R) was supported by the Federal Aid in Sport Fish Restoration Act (P.L. 81-681, Dingell-Johnson/Wallop-Breaux).

EXECUTIVE SUMMARY

A filtering performance bioassay was developed for the fingernail clam, *Musculium transversum*, a dominant bottom-dwelling organism in many waters of the midwestern United States, and a key link in food chains leading from organic matter in water and sediment to fish and ducks valued by humans. The bioassay was used with a battery of standard bioassays to assess the toxicity of porewaters obtained from sediments of the Illinois River and its associated canals (known collectively as the Illinois Waterway), where fingernail clams and other benthic macroinvertebrates died out in 1955-1958 and have not recolonized, despite the availability of seed populations in tributaries and isolated refugia within the river. Inhibition of filtering performance was easily measured with relatively simple equipment available in most laboratories and proved to be directly related to the concentration of a reference toxicant, sodium cyanide. The filtering response of *M. transversum* was consistent with the mortality response of a standard reference zooplankter, *Ceriodaphnia dubia*: both organisms exhibited no response to porewaters obtained from sediments from the lower Illinois River or from a reference site on the Upper Mississippi River, whereas porewaters from 7 of 13 upstream sites were toxic to *C. dubia* and 12 of the sites inhibited filtering performance of the clam. The responses of the clam and zooplankter were inconsistent with the responses of standard reference organisms, a freshwater alga (*Selenastrum capricornutum*) and a marine bacterium (*Photobacterium phosphoreum*), which were actually stimulated by some porewaters that were toxic to the clam and zooplankter. In view of the great physiological differences among plants, bacteria, and animals, this result was not too surprising; e.g., ammonia is toxic to aquatic animals at concentrations that can be used as a nitrogen source by plants and some bacteria.

The toxic porewaters were treated to remove certain classes of toxicants, then retested for toxicity with *C. dubia*. Based on these tests and chemical analyses of the porewaters, the toxicity in the upper Illinois Waterway is attributable largely to ammonia, with some marked local toxicity attributable to petroleum-based hydrocarbons.

During the course of this study *M. transversum* recolonized some areas in the upper Illinois Waterway where it had been absent and declined at the reference site and several other places on the Upper Mississippi River. The baseline filtering rates of clams from different sources varied, probably depending on their previous exposure to stress, including ammonia, in the waterways. Clams from some sources on the lower Illinois River and the Chicago waterways either did not respond or were actually stimulated by added ammonia concentrations of up 0.09 mg/l (un-ionized NH_3-N), indicating that they either were too stressed to respond or had been exposed to ammonia long enough to have been selected for ammonia tolerance. In contrast, added ammonia significantly inhibited the filtering of clams from the reference site in the Upper Mississippi River and two sites in the Chicago Sanitary and Ship Canal. In the short span of two months, we observed abundant clam populations disappear at some sites in the Chicago waterways, leaving only dead shells. Although a general recovery in fingernail clam populations in the Illinois Waterway does seem to be underway, this recovery apparently is set back by episodes of sediment toxicity. An investigation similar to the one reported here should be undertaken in the Upper Mississippi River where clam populations at several sites have undergone sharp fluctuations recently.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

Index to Federal Aid Jobs

Job Number	Job Title	Sections, Tables, Figures	
		Methods	Results
1	Toxicity screening	Section 2.2 Figures 2.1, 2.2, 2.3 Table 2.1	Section 3.3 Figure 3.3
2	Toxicant fractionation and testing	Section 2.9.3 Figure 2.5	Section 3.4 Table 3.2
3	Report preparation	This completion report	

ACKNOWLEDGEMENT

Many people contributed to this project. Dr. Anthony A. Paparo, Department of Zoology and School of Medicine, Southern Illinois University at Carbondale, did much of the early development of a variety of methods for assessing responses of clams and mussels to contaminants. Dr. Philippe Ross, who at the time was in the Center for Aquatic Ecology at the Natural History Survey in Champaign-Urbana, assisted Dr. Paparo in preparing the initial proposal. Ms. Diane Dillon, Mr. Jeffrey Arnold, and Mr. Kurt Pemberton provided valuable technical assistance at Western Illinois University, as did Ms. Louann Burnett at the Natural History Survey in Champaign, and Mr. K. Douglas Blodgett at the Natural History Survey's Long Term Resource Monitoring Station in Havana. Mr. Arnold assumed much responsibility for running bioassays and analyzing results toward the end of the project period. Mr. Blodgett wrote the LOTUS program for converting total ammonia to un-ionized ammonia and prepared the maps of the study area and several of the figures used in this report. Ms. Camilla Smith provided secretarial assistance at the River Research Laboratory of the Stephen A. Forbes Biological Station in Havana. The research could not have been done without the laboratory facilities, office space, and equipment provided by Dr. Richard V. Anderson and the Department of Biological Sciences at Western Illinois University--to both we express our great appreciation. We also thank the following people at the Illinois Department of Conservation: the project officer, Mr. William Bertrand, Administrator of the Streams and Rivers Program; Mr. Larry Dunham, Administrator Research and Operations; and Mr. Michael Sweet, Federal Aid Coordinator in the Division of Resource Management Finance. The Acting Head of the Center for Aquatic Ecology of the Illinois Natural History Survey, Dr. Daniel Soluk, also reviewed the report. Finally, this research would not have been done without the grant from the Federal Aid in Sport Fish Restoration Program, administered by the Region 3 U.S. Fish and Wildlife Service office in Twin Cities, MN.

1.0 INTRODUCTION

1.1 Importance of Fingernail Clams and this Research

Fingernail clams (family Sphaeriidae) are dominant bottom-dwelling animals in many waters of the midwestern United States. They are found in major rivers (Gale 1969), lakes (Emmling 1974), and bottomlands (Hubert 1972). They are key links in food chains leading from nutrients in water and mud to fish and ducks which are utilized by humans, including the highly-valued channel catfish, *Ictalurus punctatus*. Fingernail clams filter algae, bacteria and organic matter from water. Because the clams are small (<15mm or 0.6 in. long when full-grown) in comparison to mussels (family Unionidae), they are readily consumed by benthivorous fish. One species of fingernail clam, *Musculium transversum*, is especially important as a food item for fish (Ranthum 1969 and Jude 1968 and 1973) because it occurs at densities up to 100,000/m^2 or 83,600/yd^2 (Gale 1969), has a fragile, easily-masticated shell, and contains 13% protein and 2% fat (dry weight basis, Thompson and Sparks 1978). Also, *Musculium transversum* has been used as an indicator species for the benthic food base, representing other small mollusks, including snails, as well as mayflies and other burrowing aquatic insects that were virtually eliminated from certain reaches of the Illinois River by 1958 (Sparks 1984; Paloumpis and Starrett 1960).

Musculium transversum has not recolonized the Illinois River in its former numbers since 1958, despite the fact that seed populations are available in tributaries and the clam is capable of quickly repopulating an area because it has a very short life cycle--33 days in midsummer (Gale 1969). Our working hypothesis was that if we can find out what prevents the fingernail clam from recolonizing portions of the Illinois River where it was formerly abundant, we will have a strong indication of what killed the other species. Once the inimical factors are identified, they could perhaps be eliminated or controlled, so that the benthos of the river would recover, to the benefit of benthivorous fishes and diving ducks.

Declines of fingernail clams have not been limited to the Illinois River. Wilson et al. (unpublished manuscript) concluded that populations of fingernail clams declined significantly in five of eight navigation pools on the Upper Mississippi River for which historical data existed, and warned that these decreases could signal a large-scale deterioration in the health of this ecosystem. Results of research on clam declines in the Illinois River might help explain or even forestall similar declines in other rivers, such as the Upper Mississippi River.

1.2 Relationship to Other Research

Previous research demonstrated that the inimical factor was associated with sediment, rather than with the water itself, because fingernail clams survived in Illinois River water that was filtered to remove sediment (Sparks, Sandusky and Paparo 1981), and bulk sediments obtained from backwaters and floodplain lakes along the river were toxic, as measured by a clam gill bioassay (Sparks, Sandusky and Paparo 1983; Blodgett et al. 1984).

In 1988, we obtained funding from the Illinois Department of Energy and Natural Resources and the Illinois Environmental Protection Trust Fund to use a battery of standard bioassays to assess the pattern of toxicity in the river sediments and, if possible, to identify the toxic agents by treating the sediments to remove certain contaminants and then reassaying them to check for reductions in toxicity. The USEPA (Mount and Anderson-Carnahan 1988 and 1989; Mount 1988) refers to these procedures as Toxicity Identification Evaluation (TIE). The pattern of toxicity might indicate potential sources: e.g., if toxicity increased upstream, the Chicago urban area would be suspect. If toxicity increased below major tributaries that drain agricultural land in the central part of the state, then agricultural sources would be suspect. The clam gill bioassay is not a standard bioassay and so was not included. The purpose of F-94 was to use the fingernail clam bioassay in conjunction with the standard bioassays, to insure that the results were relevant to the problem of the fingernail clam decline and subsequent failure to recolonize. It is also useful to know whether the standard reference organisms are in fact good surrogates for key organisms that are important in particular aquatic ecosystems.

1.3 History of the F-94 Project

The clam gill assay was developed by Dr. Anthony Paparo, Department of Zoology and School of Medicine, Southern Illinois University at Carbondale. He became incapacitated during the course of the project and could no longer continue the bioassays. His bioassay procedure requires special equipment and skills because it involves microsurgery on the small clams and microscopic observation of particle transport rates and rates of beating of cilia on the exposed gills of the clams. The project was amended to develop a much simpler functional assay to substitute for the more complex technique, and the location of the work was shifted from Southern Illinois University to Western Illinois University in Macomb. The simpler procedure measures the ability of intact clams to filter yeast suspensions (which serve as food for the clams) from water, following exposure to test solutions. Both methods are described in this report because some interesting results were obtained with the original method.

Two other surprises occurred during the project, one pleasant and one unpleasant, that required modifications to our original plans. The unpleasant surprise was a collapse in fingernail clam populations and persistent low numbers during the period 1988-1992 in Pool 19 of the Upper Mississippi River, which had always been the source of our bioassay clams and of our supposedly uncontaminated control sediment. For a time we could not obtain enough clams to run bioassays, until we located another source, but with much lower densities, in Swan Lake on the lower Illinois River. The pleasant surprise was the reappearance of fingernail clams in several locations in the Illinois Waterway including portions of the canal system in and near Chicago. Since clams reappeared in some locations where our initial results had indicated toxicity, we ran a series of bioassays with stocks of clams from different parts of the river to determine whether some stocks were more tolerant of toxic sediments than others, perhaps having undergone

selection for resistance through a long history of exposure to local toxicants.

1.4 Objectives

In summary, the objectives of the project, as modified by the circumstances above, were: (1) to develop a simple functional bioassay for the fingernail clam, *Musculium transversum*, (2) to compare the new bioassay to the previous technique (the gill assay) and to bioassays with standard reference organisms, (3) to use the bioassay, in conjunction with the standard bioassays, to assess the pattern of toxicity in sediments of the Illinois River, (4) to use the bioassay, again in conjunction with standard bioassays, to identify toxic agents in the sediments, and (5) to determine whether stocks of clams from different parts of the river were differentially sensitive to the toxic agents. Objectives (1), (2), (3), and (5) were met. Objective (4) was partially met, by testing treated (5 treatments to reduce toxicity) sediment porewater from one location on fingernail clams, as well as on a standard reference organism, the water flea, *Ceriodaphnia dubia*, whose responses more closely paralleled those of the clam than any of the other three reference species we tested. The water flea was used for all the other toxicity identification procedures because the new clam assay was not developed as quickly as we had hoped and because of additional delays in finding a new source of clams following the decline in our original source population in the Upper Mississippi River.

2.0 METHODS

2.1 Site Description

Today's Illinois Waterway is approximately 327 miles (526 km) long connecting Lake Michigan and the Chicago-Joliet metropolitan area with the Mississippi River and the agricultural heartland, near Grafton, Illinois (Figure 2.1). The headwaters are in the highly industrialized Chicago area where the flow of the Chicago River was reversed to carry wastes away from Lake Michigan into the Illinois River via the Chicago Sanitary and Ship Canal and the downstream portion of the Des Plaines River (Figure 2.2). The Calumet Sag Channel enters the Sanitary and Ship Canal near Lemont. The Illinois River proper begins with the confluence of the Des Plaines and Kankakee rivers, and flows through a predominantly agricultural drainage, although the industrial city of Peoria is situated approximately mid-way along the waterway.

Locations on the waterways are designated by river mile as recorded in river charts prepared by the U.S. Army Corps of Engineers (1987) and by markers along the waterways, starting with mile 0.0 at the confluence with the Mississippi and proceeding upstream to Chicago. The following abbreviations are used in the text, figures, and tables to identify reaches of the waterway, and stations are identified by reach abbreviation and river mile:

IR	Illinois River proper
DP	Des Plaines River
CS	Calumet Sag Channel
SS	Chicago Sanitary and Ship Canal
CR	Chicago River

The one reference station on the Upper Mississippi River is located 377.0 miles above the confluence with the Ohio River and is designated MR 377.0. The locations of the sample stations are given in Table 2.1 and Figures 2.1 and 2.2. In accordance with Corps of Engineers terminology, the designation "left bank" or "right bank" assumes the observer is facing downstream.

2.2 Sampling Design

Nineteen sampling stations were established throughout the Illinois Waterway (Figures 2.1 and 2.2). Samples were collected from 15 stations from November 1989 to June 1990, and from all 19 stations from November 1990 to June 1991 (Table 2.1).

2.3 Sample Collection Procedures

2.3.1 Sediment Collection. It is important to limit the disruption of the sediment so that toxicity evaluations are conducted under conditions that closely match the *in situ* conditions (ASTM 1991). The most appropriate sediment sampling device is study specific. Sediment corers generally disrupt the sediment little but collect a limited sample volume (ASTM 1991). This study employed a battery of bioassays as well as the TIE procedures, all of which used sediment

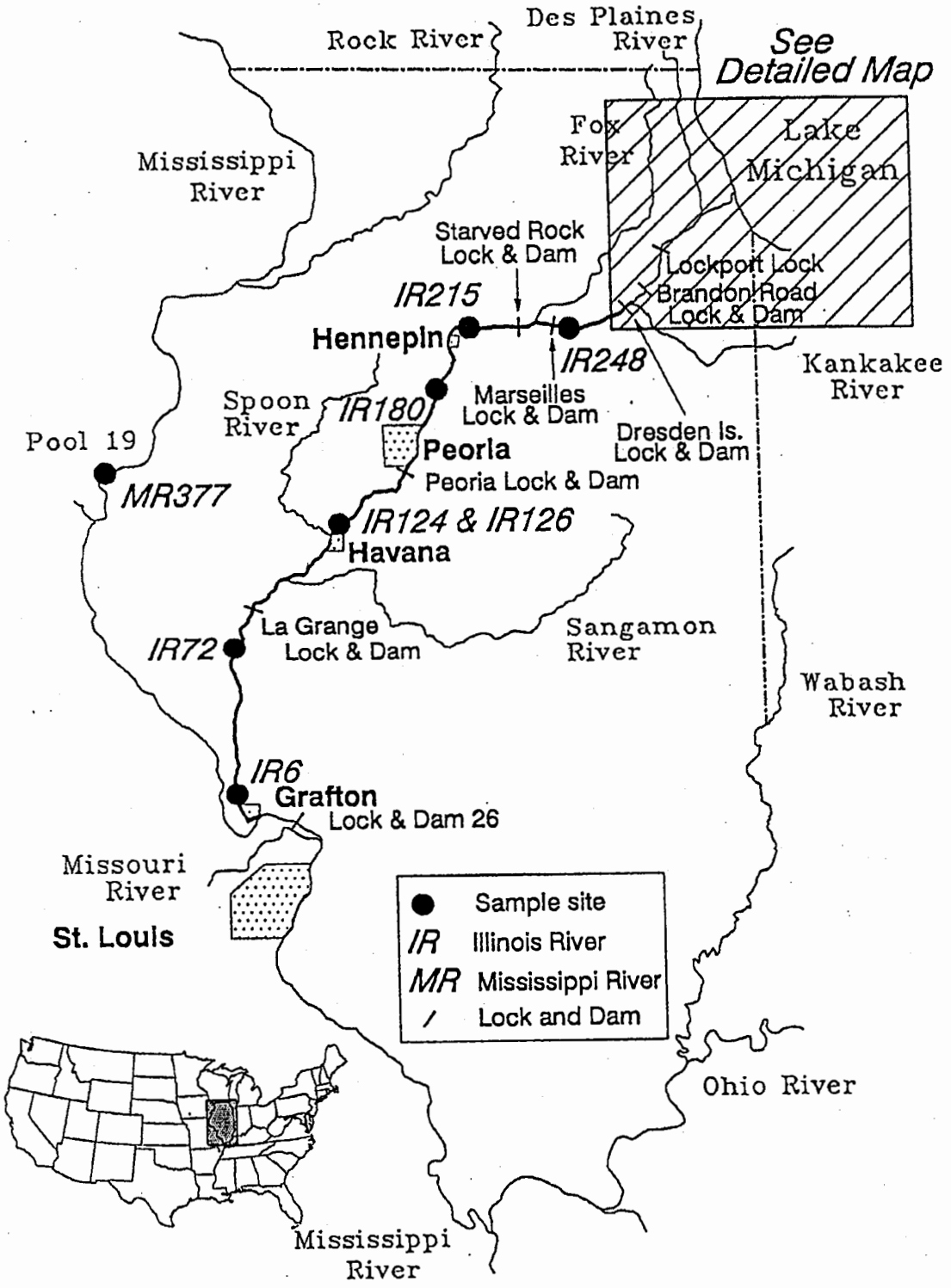


Figure 2.1. Location of sediment sampling stations on the Illinois Waterway. Stations are identified according to river miles: Illinois River miles (IR) start at Grafton at mile 0.0 and proceed upstream to Chicago. A reference station was established on the Mississippi river (MR), 377 miles above the confluence with the Ohio River.

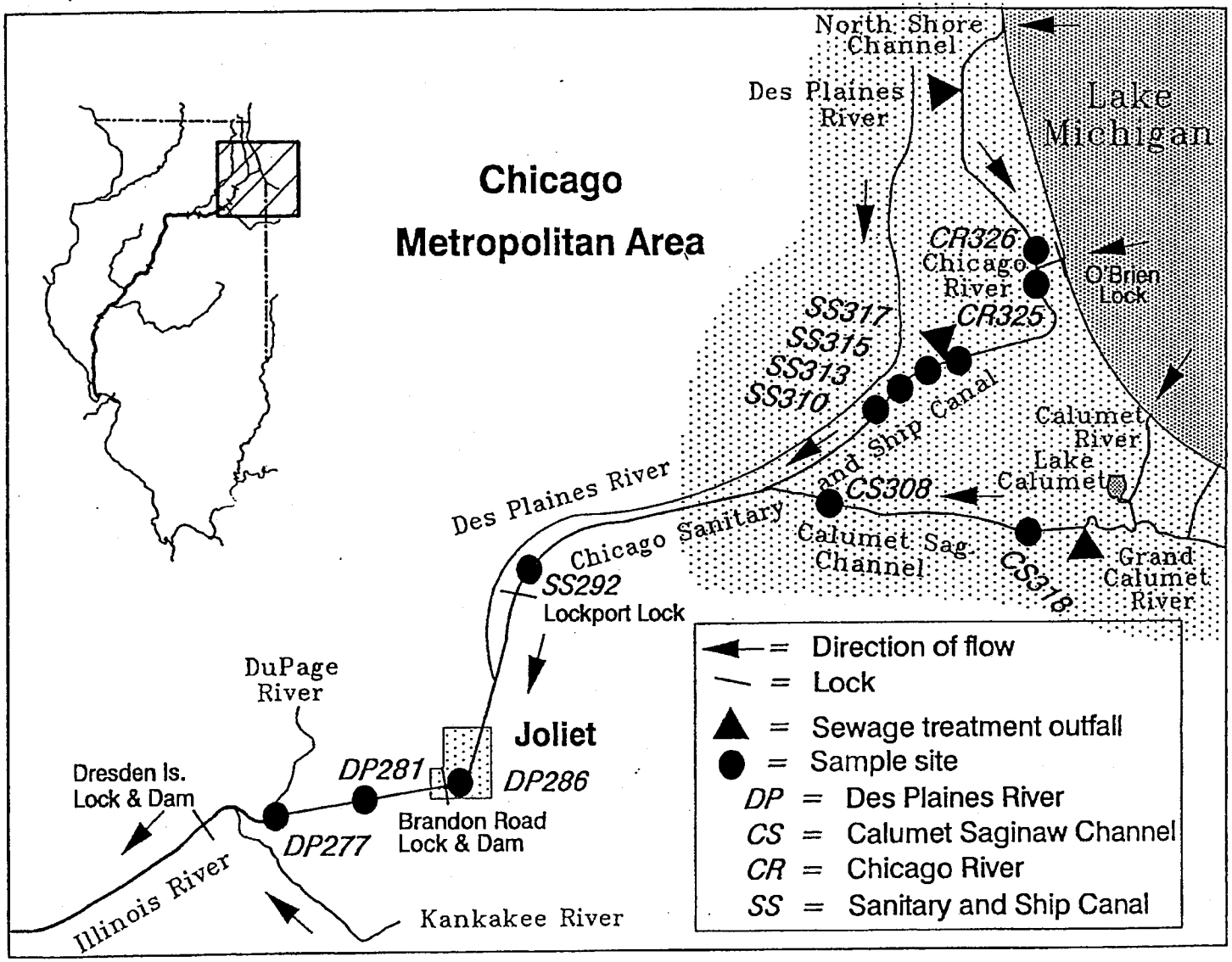


Figure 2.2. Location of sediment sampling stations in the Chicago-Joliet area. Stations (black circles) are identified according to distance (in miles) upstream from the confluence with the Mississippi River at Grafton, and according to the name of the reach. DP = Des Plaines River. SS = Chicago Sanitary and Ship Canal. CS = Calumet Sag Channel. CR = Chicago River. The location of the major sewage treatment plant outfalls in the Chicago area are noted: NSTP = northern sewage treatment plant, SWTP = southern sewage treatment plant, and CTP = Calumet treatment plant. Arrowheads indicate usual direction of flow in the waterways.

Table 2.1. Location of sampling stations.

River Mile	Description
CR 326.4	North Branch of Chicago River at Michigan Avenue Bridge
CR 324.8	South Branch of the Chicago River at Harrison Street Bridge
CS 318.5	Upstream of Division Street Bridge on Calumet Sag Channel
CS 307.4	Upstream of 104th Street Bridge on Calumet Sag Channel
SS 317.0	~5 m (16 ft) from left bank
SS 315.3	~25 m (82 ft) from left bank
SS 313.0	~2 m (6.5 ft) from right bank downstream of Route 171 Bridge
SS 310.0	~10 m (33 ft) from left bank upstream from Justice Navigation Light
SS 292.2	10 m (33 ft) upstream of sunken barge and 30 m from right bank
DP 286.3	Left bank ~300 m (984 ft) upstream of Brandon Road Lock and Dam
DP 281.1	~30 m (98 ft) from left bank across from Olin Chemical
DP 277.0	Upstream of Du Page River Daymark ~500 m (1,639 ft) from right bank
IR 248.2	~100 m (328 ft) upstream of Ballards Island
IR 215.0	Center of Turner Lake
IR 180.0	Upper Peoria Lake, south of Chillicothe
IR 125.5	SE Corner of Lake Chautauqua
IR 72.0	Center of Meredosia Lake
IR 6.0	Entrance to Swan Lake
MR 377.0	Montrose Flats, Pool 19, Mississippi River

Note: The Illinois Waterway includes the Illinois River (IR), Des Plaines River (DP), Chicago Sanitary and Ship Canal (SS), Chicago river (CR), and Calumet Sag Channel (CS). The mileages start at IR 0.0 at the confluence with the Mississippi and proceed upstream to Chicago. Mileages on the Upper Mississippi River (MR) start at the confluence with the Ohio. "Right" and "left" assume the observer is facing downstream. m = meters.

porewater. The volume of porewater needed for this work made the use of sediment corers impractical. We used a 25.4-cm (10-inch) Ekman dredge that works well in the soft to semi-soft sediments that characterize the Illinois Waterway and collects a relatively large sample volume (ASTM 1991).

The sampler was rinsed with river water at the site prior to sediment collection. The sample was placed in prewashed (Biosoap wash, ultrapure water rinse) high density polyethylene containers. High density polyethylene containers are relatively inert and are optimal for samples contaminated with a variety of chemicals (ASTM 1991). The containers were filled completely to achieve zero sample head space. Sample containers were placed on ice as soon as possible following collection (never exceeding 2 hours). Samples were transported to the laboratory and stored at 4°C (39.2°F) for no more than two weeks.

2.3.2 Extraction of Sediment Porewater. We used sediment porewater in our toxicity tests. Numerous studies (Adams, Kimerle and Mosher 1985; Swartz et al. 1985; Knezovich and Harrison 1988; Connell, Bowman and Hawker 1988; Swartz et al. 1988, Di Toro et al. 1992) have shown that porewater is an appropriate surrogate for bulk sediment. Porewater can be collected from sediment samples by several methods: centrifugation, squeezing, suction, and equilibrium dialysis (ASTM 1991). Centrifugation is generally used if large volumes of porewater are required (Edmunds and Bath 1976). Constituents such as salinity, dissolved inorganic carbon, ammonia, sulfide, and sulfate are generally not affected as long as oxidation is prevented; however, dissolved organic carbon (DOC) and dimethylsufide may be significantly reduced using this method (Howes, Dacey and Teal 1985). Sediment porewater was extracted by centrifugation at 4000 g (g = the acceleration due to gravity) at 4°C (39.2°F) for 45 minutes. The supernatant porewater was siphoned through a Nitex 110-mesh screen and stored with zero head space at 4°C (39.2°F) in a decontaminated cubitainer for a maximum of 1 week. The time from collection to testing ranged from 1 to 6 days, and averaged 2.6 days for all sediments.

2.3.3 Collection of Surface Water. Surface water samples were collected just prior to collection of sediment. Surface water was collected from approximately mid-depth in the water column using a Van Dorn sampler. Samples were placed in pre-cleaned cubitainers and immediately placed on ice. Surface water samples were stored at 4°C (39.2°F) for a maximum of one week.

2.4 Chemical Analyses

Routine chemical measurements were taken on both surface water and porewater samples. Samples were brought to ambient temperature (20-24°C, 68-75°F) prior to making the following measurements in the laboratory:

SURFACE WATER	PORE WATER
Dissolved Oxygen	Dissolved Oxygen
pH	pH
Conductivity	Conductivity
Alkalinity	Alkalinity
Hardness	Hardness
Total Ammonia-N (ammonia measured as nitrogen, N)	Total Ammonia-N
	Total Cl (chlorine)
	H_2S (hydrogen sulfide)
	Sulfide

Dissolved oxygen was measured using a standard Y.S.I. Model 57 oxygen meter with a Y.S.I. Model 5739 probe. Temperature and pH were measured using a Jenco Microcomputer pH-Vision 6071 pH meter with a temperature-compensating Ross electrode filled with Ross reference electrode filling solution #81-00-07. The pH meter was calibrated with Cole-Parmer pH 4.01 and 7.00 standard buffer solutions before use. Specific conductance was measured using a Y.S.I. Model 35 Conductance Meter with a Y.S.I. Model 3401 probe. Total alkalinity was measured using the ASTM (1982) standard titration method. Fifty ml (1.5 fl oz) of sample water was stirred by a magnetic stirrer at medium speed while the sample was titrated with 0.02 N H_2SO_4 to a pH of 3.7. Milliliters of titrant were multiplied by 20 to calculate mg/l total alkalinity as $CaCO_3$. Total hardness, as $CaCO_3$, was measured by the Hach burette method (Hach 1985) adapted from the EDTA titrametric method of APHA (1976). Total ammonia nitrogen was determined using the Hach Nesslerization method (adapted from APHA 1976). The method was modified by adding 1 drop of Rochelle salt solution prior to the Nessler reagent to prevent precipitation of magnesium hydroxide in the sample cell in water samples where hardness exceeded 100 mg/l $CaCO_3$. Results are reported as mg/l total ammonia nitrogen. Total residual chlorine was determined by the DPD colorimetric method, sulfide by the methylene blue method, and hydrogen sulfide by the lead sufide method, following the Hach Water Analysis Handbook (1985), which is adapted from APHA (1976). All instrumentation was calibrated prior to testing.

We intended to calculate the fraction of the total ammonia that existed in the un-ionized state during the toxicity tests (see below) using aqueous ammonia equilibrium calculations and knowing the pH and temperature (Emerson et al. 1975). In aqueous ammonia solutions an equilibrium exists between ammonia in the highly toxic un-ionized form (NH_3) and ammonia in the relatively nontoxic ionized form (NH_4^+). The dominant factor regulating the equilibrium between the two forms is pH, with the temperature having a lesser effect. We were not able to calculate un-ionized ammonia concentrations in the toxicity tests because the pH of the porewater drifted slightly during the tests. Temperature was held constant. However, our subsequent analysis of the correlation between toxicity and total ammonia is justified because the initial pHs of the samples were similar (6.5-7.25) and all drifted in a similar manner, so the average un-ionized ammonia concentrations during the tests were some consistent fraction of the total ammonia concentrations.

Measurements of total organic carbon (TOC) were performed on bulk sediment samples. The results are expressed in percent organic carbon.

2.5 Collection and Maintenance of Fingernail Clams

Barely enough fingernail clams were obtained from Pool 19, Mississippi River (MR 377.0) at the beginning of the project to run the gill assay described below. Another source of clams eventually was located in Swan Lake on the lower Illinois River, near IR 6.0, and these were used throughout most of the project period to develop the bioassay that measured filtering performance and to test the toxicity of porewater from sediments. Finally, near the end of the project period, we collected clams from MR 365.5, IR 5.1, and two sites in the Chicago waterways (Sanitary and Ship Canal, SS 292.7; Calumet Sag Channel, CS 318.5; see Figure 2.1 and 2.2), to compare the sensitivity of clams from the different sites to ammonia. We also recorded observations on the number of live clams and dead shells at several other sites where we did not obtain clams in sufficient numbers to run bioassays.

Sediments were collected with an Ekman dredge and sieved through a wash bucket with a 500-micron screen. Clams were hand-picked from the screen and kept in a cooler with aerated river water until delivered to the laboratory. They were held in aquaria in river water and fed a suspension of the green alga, *Selenastrum capricornutum*, and a yeast-Cerophyl-trout food (YCT) that is normally used for *Ceriodaphnia dubia* (NETAC 1989b). Clams were used within 14 days of capture. They were placed in synthetic dilution water 1 hour prior to being tested. The total hardness of the dilution water was adjusted to match that of the sediment pore water, by adding the appropriate salts to increase hardness, or by dilution with distilled, deionized water to reduce hardness.

Although every effort was made to obtain clams in the field that were approximately the same size, and again to choose clams of the same size from the holding aquaria for the experiments, we were forced to use a wider range of sizes than we wished, because it took so much effort to find clams at the field sites. The shell lengths of clams used in these experiments ranged from 5.9 to 12.0 mm (0.236-0.480 in), averaging 9.0 mm (0.360 in).

2.6 Fingernail Clam Gill Bioassay.

Dr. Anthony Paparo conducted the gill assays on clams kept for at least seven days in circulating, aerated river water in an Instant Aquarium (temp. 17-20^{0} C 62.6-68^{0} F, pH 7.5) in a laboratory at Southern Illinois University in early April 1989. Before each experiment, clams 5.0-10.0 mm in length were placed in finger bowls of the same river water. The posterior adductor muscles were cut, and each gill with its branchial nerve, visceral ganglion, and a piece of adductor for support, were isolated. The ganglion/nerve/gill preparation was pinned to a rubber mat glued to the bottom of a Petri dish containing river water, and the dish was placed in a holder fastened to the adjustable stage of a microscope. Under magnification (100X), the gill was seen to consist of numerous parallel gill filaments. Three major types of ciliated cells were clearly distinguished: frontal, laterofrontal and lateral.

The rate of beating of the lateral cilia was measured. These cilia beat in such a way that metachronal waves appear to travel in opposite directions along the two sides of each gill filament.

The optical field was selected for observation by measuring 2.0 mm in an anterior direction from the visceral ganglion. A field of view contained about 50 gill filaments which were grouped for ease of observation into three vertical columns. By moving the microscope stage each gill filament was followed from its dorsal attachment at the axis to its free ventral end. Each column was subdivided into four horizontal rows, demarcated by their fixed number of interfilamentary junctions, from dorsal to ventral end. The rate of ciliary beating in beats per second was measured by synchronizing the rate of flashing of a calibrated stroboscopic light (used in place of the substage lamp) with the rate of beating of the cilia. Synchronization was achieved when the metachronal wave appeared to stand still. The rate was then read from a digital flash rate on the strobe. Measurements were made from dorsal to ventral border, and from left to right across the field: 12 sets of measurements for each gill preparation, which then were averaged.

Sediment suspensions were prepared by adding the same arbitrary volume of spoon-mixed wet sediment from each site (collected from Pool 19, Upper Mississippi River, on 21-22 March 1989, and from the Upper Illinois River 28-29 March) to one liter of standard molluscan physiological solution. The test solutions then were diluted with the physiological saline solution until they all had similar particle concentrations, as determined by counting the particles with a hemocytometer. Control suspensions were prepared to match the average particle concentration of the test solutions, only using yeast instead of sediment. Blodgett (1983) reported the following ranges in mean sediment and yeast suspensions from similar experiments:

	Particle Size μ m ($.04 \times 10^{-3}$ in)	Density mg/l (ppm)	Concentration 10^6 particles/l (1.06×10^6 particles/qt)
sediment	2.9 - 7.2	29.3 - 78.4	2.1 - 3.9
yeast	6.8 - 7.2	46.3 - 59.1	2.5 - 2.7

The suspensions were pumped across the Petri dishes containing the gill preparations via a four-channel, variable-speed pump with a flow rate of about 0.5 ml/min (.015 fl oz/min). The planetary gear mechanism of this pump ensured minimum pulsing and stable drift-free flow, permitting accurate measurement of ciliary movement. A positive displacement piston metering pump with micrometric adjustment removed the solution from the other side of the dish, thereby maintaining a continuous flow of solution across the dish. The temperature was maintained at 20^0 C (68^0) by circulating water from a constant temperature bath at 2.0 ml/min (.06 fl oz/min) through stainless steel tubing in the movable microscope platform that held the Petri dish. Dissolved oxygen remained at 8.0 ppm during the exposure periods, and the pH was monitored and adjusted to 7.5 by adding acid or base, if necessary.

Ten gill preparations were used for each sample tested: five controls (exposed to yeast suspensions) and five experimental

preparations (exposed to the sediment suspensions). Measurements were made prior to introduction of the suspensions and again after 30 minutes of exposure to the suspensions. The measurements were averaged across the five animals in each group, and the results expressed as a percentage increase (stimulation) or decrease (inhibition) in ciliary beating rates of the experimental animals relative to the controls. In 20 previous control tests with equivalent yeast suspensions the gill preparations showed a net change of 0 ciliary beats/sec $\pm$ a standard deviation of 1.25 during the 30-min. exposure period (Blodgett et al. 1984). Therefore, a reduction in the ciliary beating rate of the test gills was regarded as a response to toxicity in the sediments and not to the particle concentrations.

2.7 Fingernail Clam Filtering Bioassay.

2.7.1 Rationale and General Procedure. The fingernail clam filtering assay developed in this study is based on observations by Aldridge, Payne and Miller (1987), Sparks and Sandusky (1983), Sparks, Sandusky and Paparo (1981), and Anderson, Sparks and Paparo (1978) that stresses, including toxicants, impair the ability of bivalves to filter particles from water (including food particles, such as yeast, on which the clams feed). Only the 1990-1991 porewater samples were evaluated using this assay because it was not fully developed until late 1990. A detailed description of the general procedure follows.

Fingernail clams are first exposed to the porewater sample for one hour. They are then removed from the test solution and given a filtering performance test, which consists of placing them in a yeast suspension in dilution water (10% by weight, based on dry weight of yeast) and allowing them to filter for one hour (Figure 2.3). Two controls are used: the first consists of the yeast suspension alone and is used to determine the change in concentration due to settling of the yeast. The second control determines the baseline filtering rate of clams exposed for 1 hour in clean, uncontaminated water.
The yeast concentrations are measured indirectly at the beginning and end of the filtering period, by measuring light transmission in water samples from the test chambers with a spectrophotometer, then using a regression equation that relates light transmission to measured yeast concentrations:

Y = yeast concentration (mg/l)

T = % transmittance of light

$$Y = 676.378 - 6.788\ T$$

The filtering rates of the exposure and control tests are determined by taking the initial yeast concentration minus the final concentration minus the amount settled divided by the weight of the test organisms.

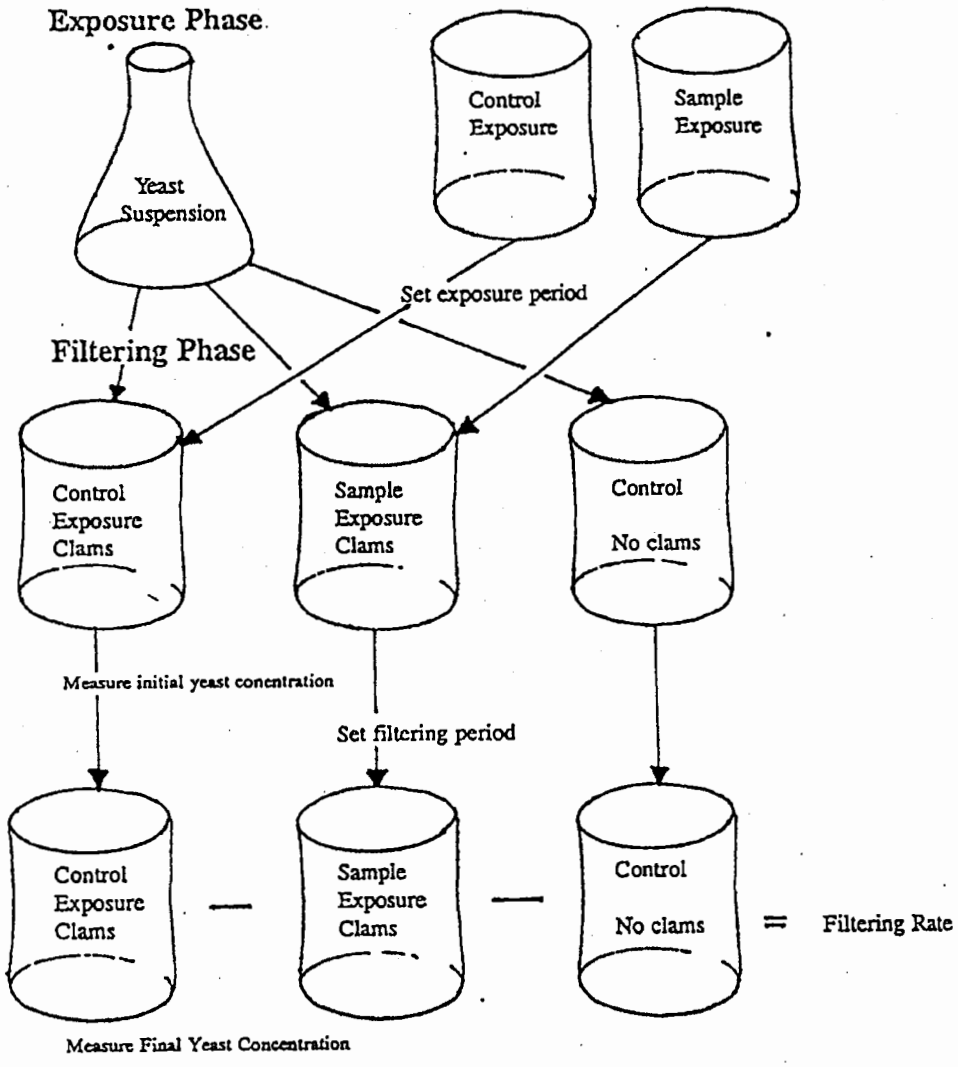


Figure 2.3. Steps in fingernail clam filtering bioassay.

Filtering rates are expressed as the concentration of yeast filtered per unit weight of organisms per unit time.

C_i = initial concentration of yeast

C_f = final concentration of yeast

W = live weight of clams, in g (grams), x 0.035=oz

C_s = change in yeast concentration due to settling

$$\frac{C_i - C_f - C_s}{W}$$ = filtering rate in mg (milligrams) yeast/g clam/hour (mg x 3.527×10^{-5} = oz)

The organism weights are for whole live animals, with shells, blotted dry with Chem Wipes, a tissue type of absorbent material. Five clams were used in each test chamber.

2.7.2 Assessment of Response. The filtering rate of clams exposed to the test solutions is then compared to the control to determine the degree of response. Since there is natural variation in the filtering rate of healthy clams, we decided to calculate a threshold value for a change in filtering rate that we would consider to be beyond the range of normal variation. We took the maximum range of variation in filtering rate observed in all the control trials and added two times the standard deviation. This threshold amounted to 10.6% of the mean control value, so filtering rates would have to decline more than 10.6% in relation to the control before the test solution would be regarded as toxic (inhibitory). Likewise, the filtering rate would have to increase more than 10.6% before the test solution would be classified as stimulatory.

2.8 Reference Toxicant.

It is useful to have a bioassay with a graded response so that sampling sites can be ranked according to relative toxicity, thus revealing spatial and temporal trends that might indicate sources. "All or none" (death or survival, toxic or nontoxic) responses are less useful for this purpose. In order to determine whether filtering inhibition was proportional to toxicant concentration, we tested a reference toxicant, cyanide, over a range of concentrations (1-100 mg/l added as sodium cyanide salt) known to bracket a lethal level for fish and other standard test species.

2.9 Sensitivity of Clams from Different Sites to Ammonia.

This component was added late in the project when it became obvious that fingernail clams were reappearing in portions of the Illinois Waterway where our Toxicity Identification Evaluation (TIE) procedures had identified toxicity attributable to ammonia. Fingernail clams from the various sources were exposed to dilution water as a control and to three concentrations of ammonia in dilution water, to

determine whether clams from different sites differed in their tolerance for ammonia. The ammonia was added as ammonium chloride, measured in the test solutions as described in section 2.3, and the un-ionized ammonia nitrogen, NH_3-N, concentrations calculated and reported.

2.10 Reference Bioassays.

2.10.1 Reference Species and Responses. We compared the response of fingernail clams, *Musculium transversum*, to the responses of four standard reference species, as determined in the DENR project (Sparks, Ross and Dillon 1992). The standard bioassays employed the following organisms: the marine bacterium, *Photobacterium phosphoreum* (Microtox™), the freshwater alga, *Selenastrum capricornutum*, the rotifer, *Branchionus calyciflorus*, the daphnid, *Ceriodaphnia dubia*, and the sphaerid clam, *Musculium transversum*. The Microtox™ assay measures the luminescence of *P. phosphoreum* (Bulich, Greene and Isenberg 1981). Inhibition of this luminescence is considered a toxic response. The *S. capricornutum* assay measures the inhibition of photosynthetic activity of an algal culture as a measure of toxicity (Ross, Jarry and Sloterdijk 1988). The rotifer assay is a mortality test (Snell and Personne 1989). The *C. dubia* assay was the standard USEPA (1985) acute assay (48-hour mortality). The dilution water used initially in the toxicity tests and for maintaining the organisms was 1 part Perrier™ bottled water to 9 parts distilled water passed through a Millipore Ultrapure water system. Following a contaminant scare, all Perrier water was removed from the market, so we made our own systhetic dilution water, starting with water from the Ultrapure system and adding salts (Marking and Dawson 1973).

Results of the *C. dubia* bioassay are expressed in toxicity units, as well as 48-hour LC50s, where toxicity units = 100/(48-hour LC50). The 48-hour LC50 is the percent dilution of porewater (or treated porewater) that kills 50% of the test organisms in 48 hours. For example, if a 7% solution (by volume) of porewater in dilution water is the LC50 (see site CS307.4, Table 3.1 in the Results Section), then:

7% = 48-hour LC50
100/LC50 = 100/7 = 14.3 toxic units

meaning that the toxicity in the porewater is more than 14 times the lethal dose.

2.10.2 Standardization of Responses. The results of the various assays were standardized for easier comparisons. The treatment results were divided by the control results and then 1 was subtracted from the quotient. A negative value indicates inhibition (toxicity), a positive value indicates stimulation, and 0 indicates no response (no difference

with respect to the control). If we use the fingernail clam filtering bioassay as an example:

T = test response to sample of sediment porewater

C = control response to uncontaminated dilution water

T = 3.4 mg yeast/g clam/hour

C = 6.5 mg yeast/g clam/hour

T/C = 3.4/6.5 = .52

.52 - 1.00 = -.48 A decline of 48% from the control value, a marked inhibition of the filtering ability of the clams.

2.10.3 Test Samples. A battery of bioassays, including the clam filtering bioassay, were used on raw porewater from Pool 19 of the Upper Mississippi River and from sites along the entire length of the Illinois Waterway, but concentrated in the Chicago metropolitan area (Figures 2.1 and 2.2). The purpose of this comparison was to determine whether the various bioassays consistently identified the same upstream-downstream patterns, including "hot spots" of toxicity. In addition, the clam filtering bioassay was compared to the *Ceriodaphnia dubia* bioassay, using treated porewater from a "hot spot" on the Des Plaines River portion of the waterway (DP 277.0). The purpose of the latter test was to determine how well the filtering bioassay performed within the Toxicity Identification Evaluation (TIE) protocols developed for use with *C. dubia* and other standard reference species at the USEPA's National Effluent Toxicity Assessment Center (NETAC 1989a; Mount 1988; Mount and Anderson-Carnahan 1988 and 1989).

The goal of TIE is to separate toxicants from nontoxic compounds, using sample fractionation techniques in combination with bioassays to determine which fractions contain most of the toxicity. The TIE approach consists of three phases outlined in Figure 2.4. Only samples from Phase 1 treatments were assessed with the clam filtering bioassay. Phase 1 characterizes the physical and chemical properties of the sample toxicants by altering or rendering biologically unavailable generic classes of compounds (Mount and Anderson-Carnahan 1988). After Phase I the toxicants are classified as having characteristics of cationic metals, non-polar organics, volatiles, oxidants, or substances not affected by Phase I methods. The Phase I treatments are outlined in Figure 2.5. The primary tool of Phase I is manipulation of sample pH. The questions asked are: (1) Is toxicity different at different pHs? (2) Does sample manipulation at different pHs affect toxicity? (3) Is toxicity attributable to cationic metals, such as copper or lead? (4) Is toxicity associated with oxidizing agents, such as chlorine or chloramines? The graduated pH test answers the first question and is designed to indicate a pH-dependent toxicant such as un-ionized ammonia. The second question is answered by performing the following tests at different pHs: aeration, filtration and reverse-phase, solid phase extraction (SPE) on C_{18} columns. Aeration tests determine whether toxicity is attributable to volatile or oxidizable compounds. The filtration tests indicate whether

TOXICITY-BASED TOXICITY IDENTIFICATION EVALUATION

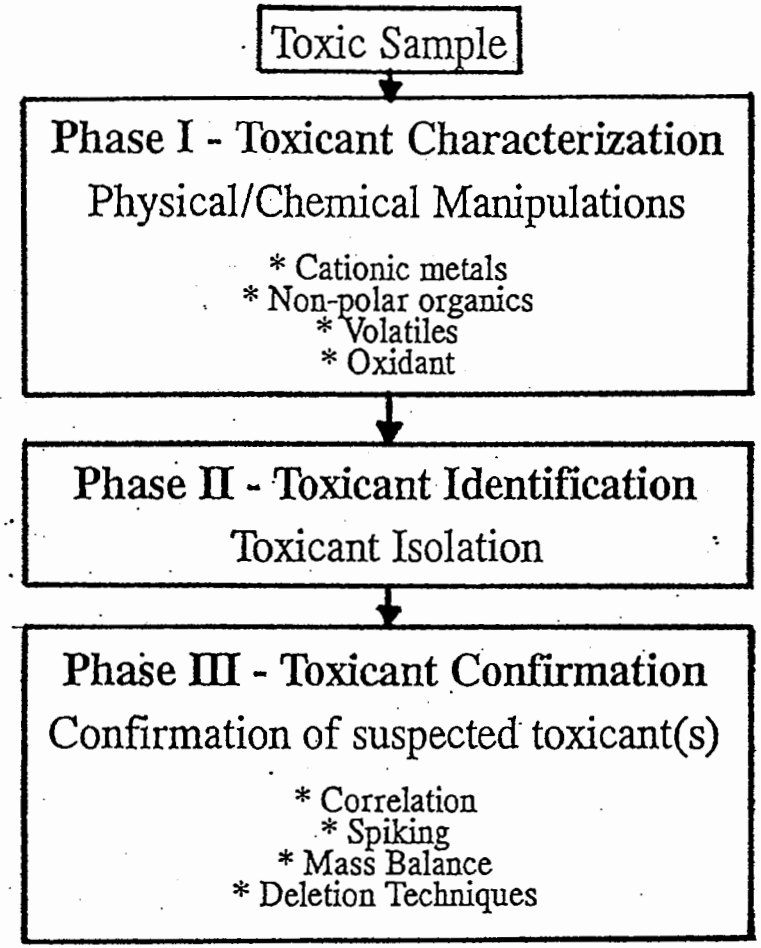


Figure 2.4. The three phases of Toxicity Identification and Evaluation (TIE) procedures.

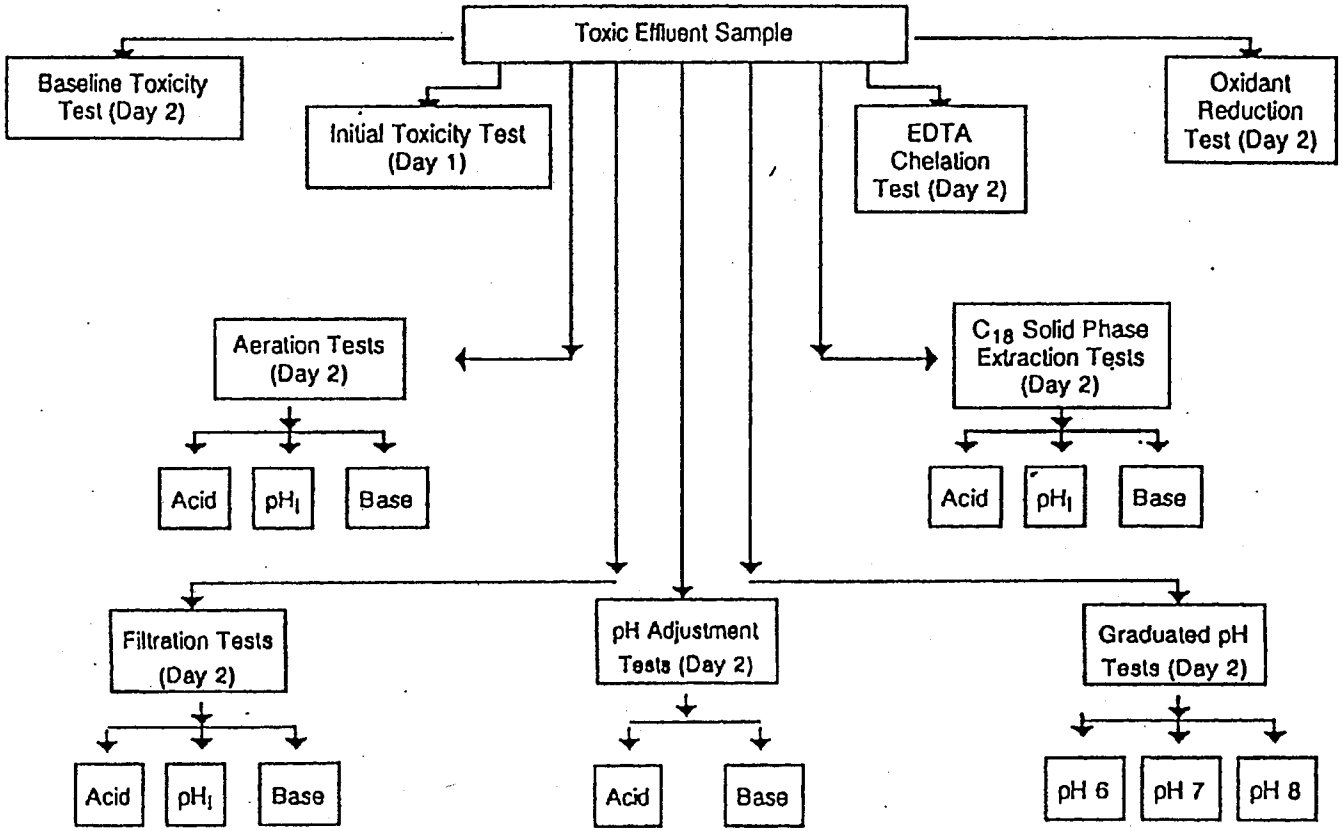


Figure 2.5. Steps involved in Phase I Toxicity Identification and Evaluation (TIE). Source: Mount and Anderson-Carnahan 1988.

toxicity is associated with filterable components. Reverse-phase SPE indicates whether toxicity is attributable to non-polar compounds. Presence of toxic cationic metals is indicated if addition of a chelating agent, ethylenediaminetetraacetic acid (EDTA), diminishes toxicity. Presence of chlorine or other oxidizing agents is indicated by a reduction in toxicity following addition of the reducing agent, sodium thiosulfate.

3.0 RESULTS

3.1 Development of the Fingernail Clam Filtering Bioassay

The filtering performance bioassay certainly required less specialized equipment and skill than the clam gill bioassays used previously (Blodgett et al. 1984; Anderson, Sparks, and Paparo 1978; Sparks, Sandusky and Paparo 1981; Sparks, Sandusky and Paparo 1983). As we had hoped, the inhibition of filtering rate was proportional to the amount of the reference toxicant, sodium cyanide, added to test solutions (Figure 3.1). We also observed that some individual fingernail clams would eject water while they were being blotted dry. If some individuals retained water within their valves when they were weighed, and others ejected it, the weight wets and hence the filtering rates per gram wet weight would be highly variable, perhaps masking real differences in response. Another problem was that our clams came from populations that were clearly under various degrees of stress from 1988 to 1991. Many organisms lose tissue mass under stress, which means that the weight of living tissue in our clams might have been below average or highly variable, while the weight of the shells and the water they enclosed would remain the same. Since it is the living tissue that does the filtering, it would be better to measure filtering rates per unit weight of tissue, preferably oven-dried (100^{0} C) tissue, to avoid any additional variation caused by differences in water retention of the tissue.

3.2 Comparison with Other Bioassays.

3.2.1 Filtering Assay Compared to Gill Assay. Both the clam gill assay and the clam filtering assay indicate toxicity in sediments in the upper Illinois Waterway, close to the Chicago area, and much less, or no toxicity in Pool 19 of the Upper Mississippi River (Figure 3.2). The filtering assay indicates no toxicity in the Illinois Waterway below River Mile 248 (Figure 3.2). Unfortunately, no samples below River Mile 286.0 were tested with the gill assay for comparison during this project, although sediment samples taken from Quiver Lake (Illinois River Mile 123.0) in November 1980 and water column samples from the main channel at Havana (Illinois River Mile 119.6) in October 1977 were toxic, as measured by the gill assay (Sparks, Sandusky and Paparo 1983 and Anderson, Sparks, and Paparo 1978). Both assays agree that toxicities in the North Branch of the Chicago River (miles 325 and 326) are lower than at some downstream stations, indicating a likely source of toxicity between mile 325 and the downstream points.

The toxicities recorded by the two methods differ in detail, however, which is not too surprising considering that bulk sediment samples collected in March 1989 were used for the gill assay and porewater from samples collected more than two years later, in 1991, were used for the filtering assay. 1988-1989 was a period of drought and extreme low flows in both the Upper Mississippi River and Illinois River, and fingernail clam populations were declining in Pool 19. Flows returned to nearly normal in 1990. The gill assay indicated nearly a 20% inhibition in response to Pool 19 sediments in 1989, whereas the

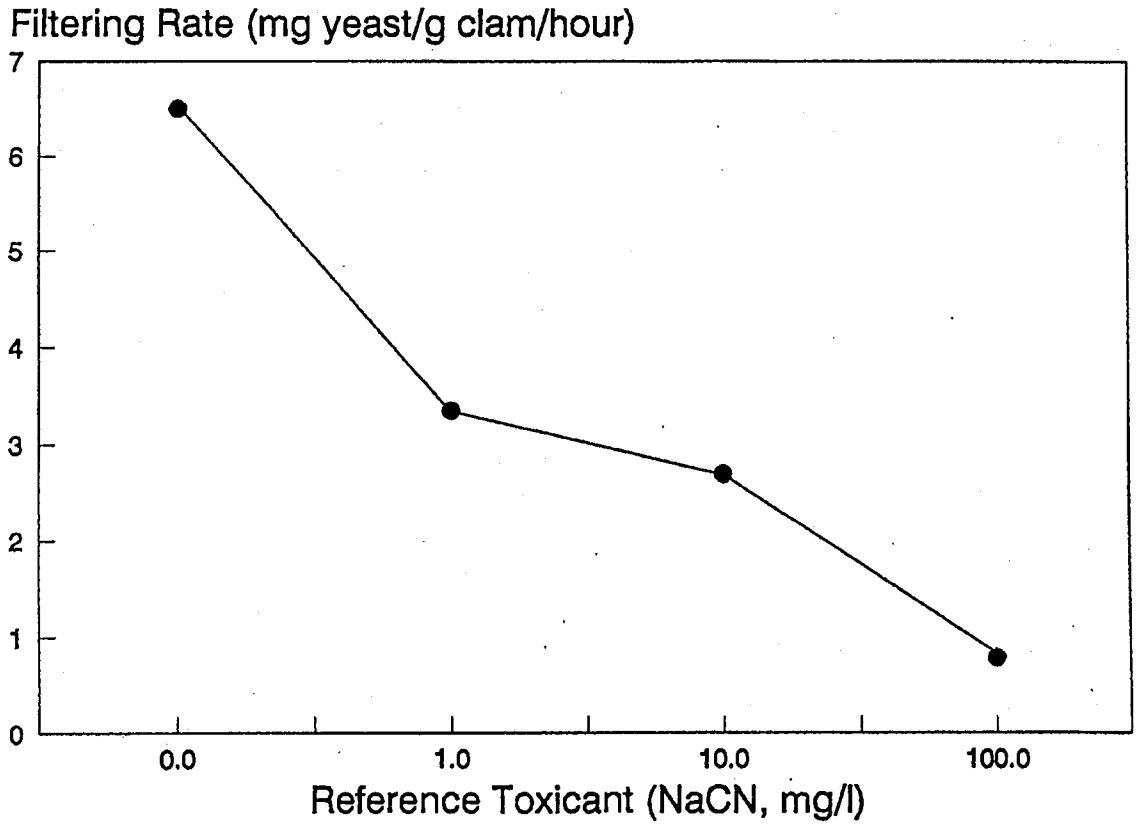


Figure 3.1. Fingernail clam filtering response to a reference toxicant, sodium cyanide (in mg/l added as salt, NaCN).

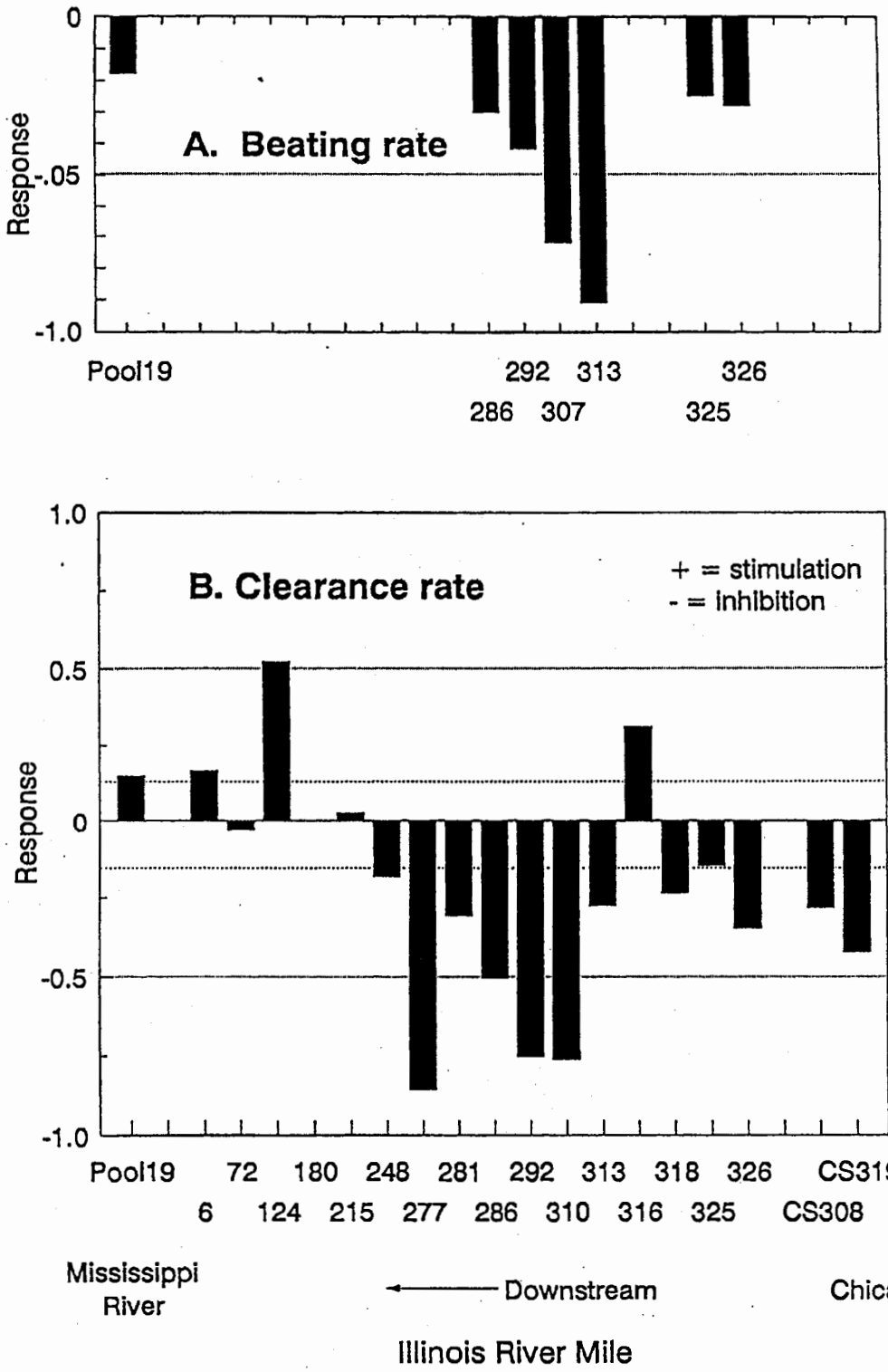


Figure 3.2. Comparison of the response of isolated clam gills (A) and the filtering response of intact clams (B) to sediments from the Illinois Waterway. the gill response is expressed in terms of the inhibition of the beating rate of cilia on the gills, in comparison to control gills maintained in uncontaminated dilution water. The gills were exposed to dilutions of bulk sediments collected in March 1989. The filtering response measures the ability of the clams to clear yeast suspensions, in comparison to controls. These clams were exposed to sediment porewater collected in 1991.

filtering assay indicated a barely significant stimulation in 1991, perhaps in response to sodium, potassium, calcium, and magnesium salts in the porewater. Anderson, Sparks and Paparo (1978) demonstrated the importance of these salts in regulating the beating of the cilia on the gills of the clams. Salts that affect the cilia are likely to affect filtering performance because the lateral cilia produce the water currents that bring food into the clam and the latero-frontal cilia act as filters. Also, the presence of organic matter in the sediment porewaters may have stimulated a feeding response in the clams, which are deposit feeders, as well as water column filterers. The most toxic site on the Illinois Waterway in 1991, according to the filtering assay, was DP 277.0, a site not tested in 1989. The most toxic site in 1989 was SS 313.0, which the filtering assay also rated as toxic in 1991, but less so than 5 sites that were 3-36 miles downstream (Figure 3.2).

3.2.2 Filtering Assay Compared to Standard Bioassays. There were marked differences in the responses of the five test organisms to sediment porewater from the same sites (Figure 3.3). Luminescence of the marine bacterium, *Photobacterium phosphoreum*, (Microtox test) was inhibited by 34% at SS313.0 on the Sanitary and Ship Canal and 32% at CS307.4 on the Calumet Sag Channel. Maximum stimulation of approximately 50% occurred at the next site upstream on the Calumet Sag Channel, CS318.5. Responses to porewaters from other sites were slight and variable, sometimes mildly inhibitory and sometimes mildly stimulatory.

Photosynthesis by the freshwater alga, *Selenastrum capricornutum*, was markedly stimulated, by a factor of nearly 2, by sediment porewaters from the mouth of Swan Lake, IR6.0, and the Sanitary and Ship Canal, SS310.0. Stimulation is an indication of nutrient enrichment; e.g., by nitrogen and phosphorus (Ross et al. 1988). The greatest inhibition, -86%, was caused by sediment porewater from Lake Chautauqua, IR125.5, although inhibition also occurred at IR72.0, IR281.1, SS313.0, SS315.3, and CS307.4.

A large percentage of the rotifers, *Branchionus calciflorus*, died in porewaters from Meredosia Lake (IR72) and Lake Chautauqua (IR125.5), but the rotifers exhibited no significant responses anywhere else (Figure 3.3).

In contrast to the microorganisms (bacterium, alga, and rotifer), the macroinvertebrates *C. dubia* and *M. transversum* were remarkably consistent in their responses to the sediment porewaters. Both organisms exhibited no inhibitory response to porewaters from the lower Illinois River or from the reference site in the Upper Mississippi River (Figure 3.3). The clam and the water flea likewise are consistent in indicating toxicity in the upper waterway. Filtering performance in the clam was inhibited starting with sediment porewaters from IR248.2 near Marseilles and water flea mortality started at DP277.0, just above the mouth of the Du Page River near the Interstate 55 bridge.

3.3 Pattern of Toxicity in the Illinois Waterway.

Toxicity in sediment porewaters, as measured by bioassays using macroinvertebrates as test organisms, increases in the upstream direction, toward the Chicago-Joliet area, indicating a likely source of toxicity in that region. Sediment porewaters from 7 of the 13 upstream

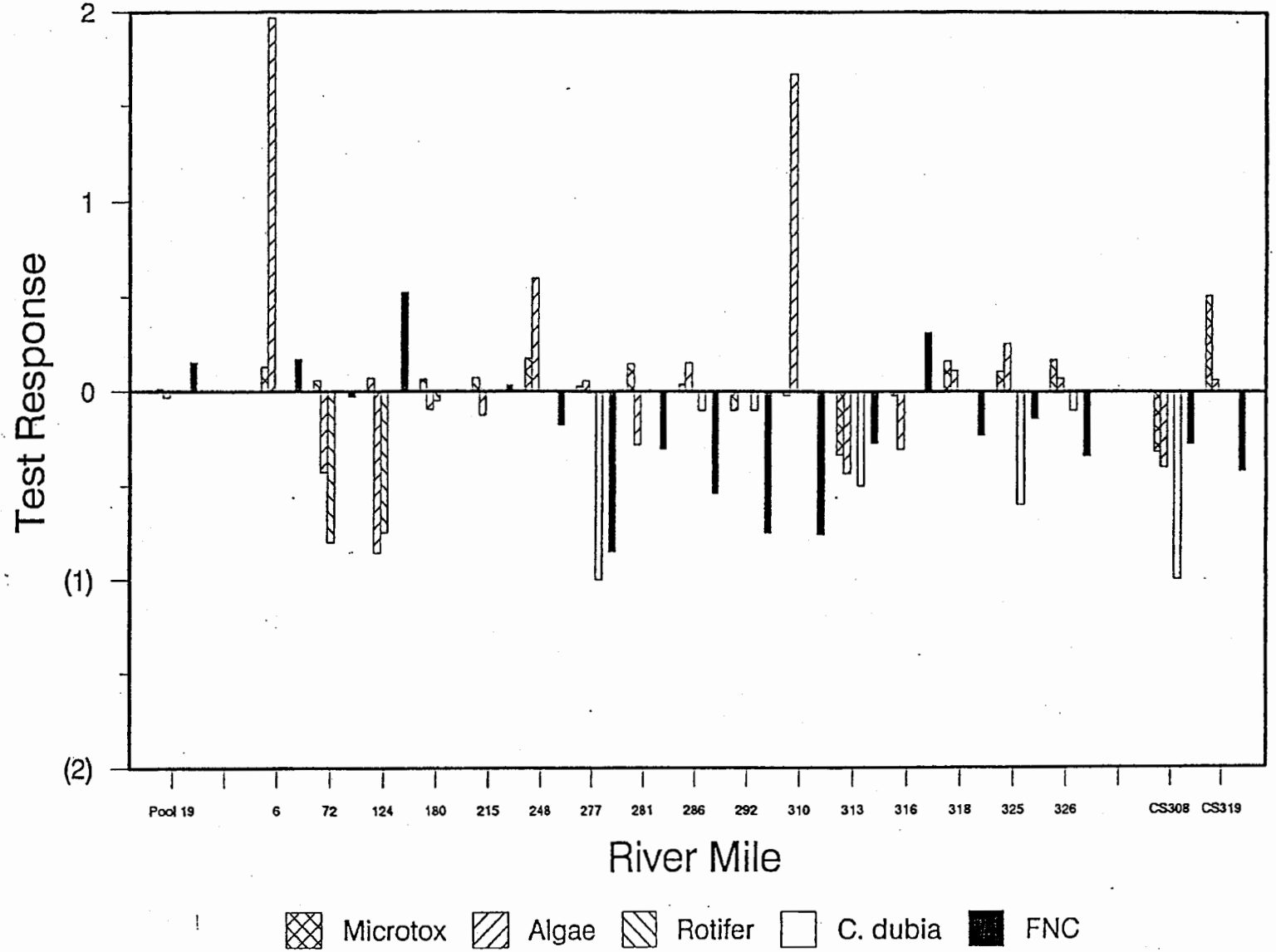


Figure 3.3. Responses of five organisms to porewaters from sediments of the Illinois Waterway and from a reference site (Pool 19 of the Upper Mississippi River). Numbers above zero represent stimulation, numbers below zero represent inhibition (toxicity). CS = Calumet Sag Channel. FNC = fingernail clams.

sites were toxic to the water flea *C. dubia*, and 12 of 13 inhibited the filtering performance of the fingernail clam (Figure 3.3). Neither organism indicated any toxicity in porewaters from the lower Illinois River or in Pool 19 of the Upper Mississippi River. The toxicity of porewater to *C. dubia* appeared to be closely related to the total ammonia concentration in the porewater in 1989 and 1990, with the exception of site DP277.0 in 1990, where factors other than ammonia may have contributed to the peak toxicity there (Figure 3.4). Toxicity to *C. dubia* correlated with total ammonia in porewater samples taken in 1990 and 1991 ($r = 0.85$, Figure 3.5). Porewaters generally had much higher total ammonia concentrations and greater conductivities than surface waters from the same sites (Table 3.1).

Porewater from site DP277.0 was most toxic to the fingernail clam, and highly toxic to the water flea, so it was subjected to TIE Phase I analysis, the results of which are described below. This was the only TIE analysis that employed both types of bioassays (*M. transversum* and *C. dubia*) and therefore is included in the results section of this report. All other TIE analyses used *C. dubia* toxicity tests and these results are reported in Sparks, Ross, and Dillon (1992).

3.4 Toxicity Identification and Evaluation (TIE).

The *C. dubia* mortality test and the *M. transversum* filtering assay both indicated that toxicity of porewater from DP277.0 was not removed by chelation with EDTA, so toxicity was not attributable to heavy metals (Table 3.2). Both tests were consistent in indicating that toxicity persisted when the bioassays were run at both lower (6.5) and higher (8.5) pHs (Table 3.2). The clam assay indicated toxicity increased at both the higher and lower pH, a result that might be caused by the presence of two toxicants whose chemical equilibria are pH-sensitive. For example, the proportion of ammonia existing in the toxic, un-ionized form (NH_3) will increase at higher pHs, and the proportion of hydrogen sulfide existing in the toxic, un-ionized form (H_2S) increases at lower pHs. Both bioassay techniques indicated that toxicity decreased from the initial baseline value upon standing or with aeration at the higher pH of 11 (Table 3.2). This result is consistent with the presence of ammonia, whose un-ionized form can be oxidized and volatilized.

Results of the two bioassays are inconsistent with each other in virtually all the other combinations of treatments. According to the *C. dubia* bioassay, virtually all the toxicity was removed by filtration or passage through the C_{18} column at a pH of 3, whereas the clam assay recorded the highest toxicity (-0.94) with the C_{18}C-pH 3 treatment and considerable toxicity even after filtering the porewater (Table 3.2). The clam assay recorded the least toxicity (-0.04) following aeration at a pH of 3, whereas there was considerable mortality in the *C. dubia* bioassay.

3.5 Ammonia Tolerance of Clams from Different Sources

3.5.1 Field Observations. In October and November 1991 we tried to obtain sufficient clams from Pool 19 of the Mississippi River, Swan Lake on the Illinois River, and from waterways in the Chicago area to assay ammonia tolerance of clams from populations that presumably had been exposed to different levels of ammonia in sediment pore water.

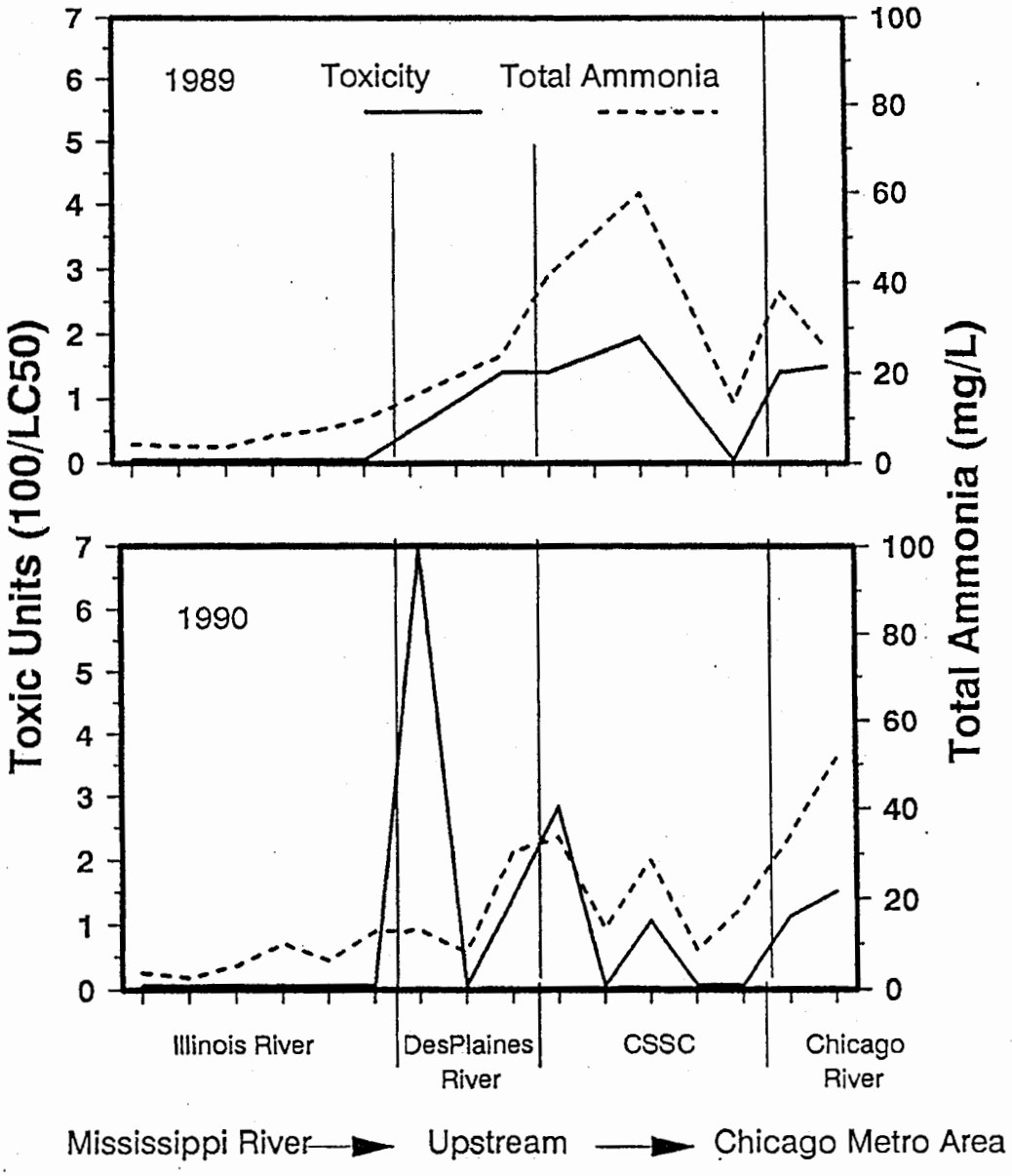


Figure 3.4. Toxicity and total ammonia concentrations in sediments of the Illinois Waterway in 1989 and 1990. Toxicity was measured with the *Ceriodaphnia dubia* bioassay. The highest toxicity observed (in the Des Plaines River portion of the Waterway, DP277.0, in 1990) was attributed to a combination of ammonia and petroleum-related hydrocarbons.

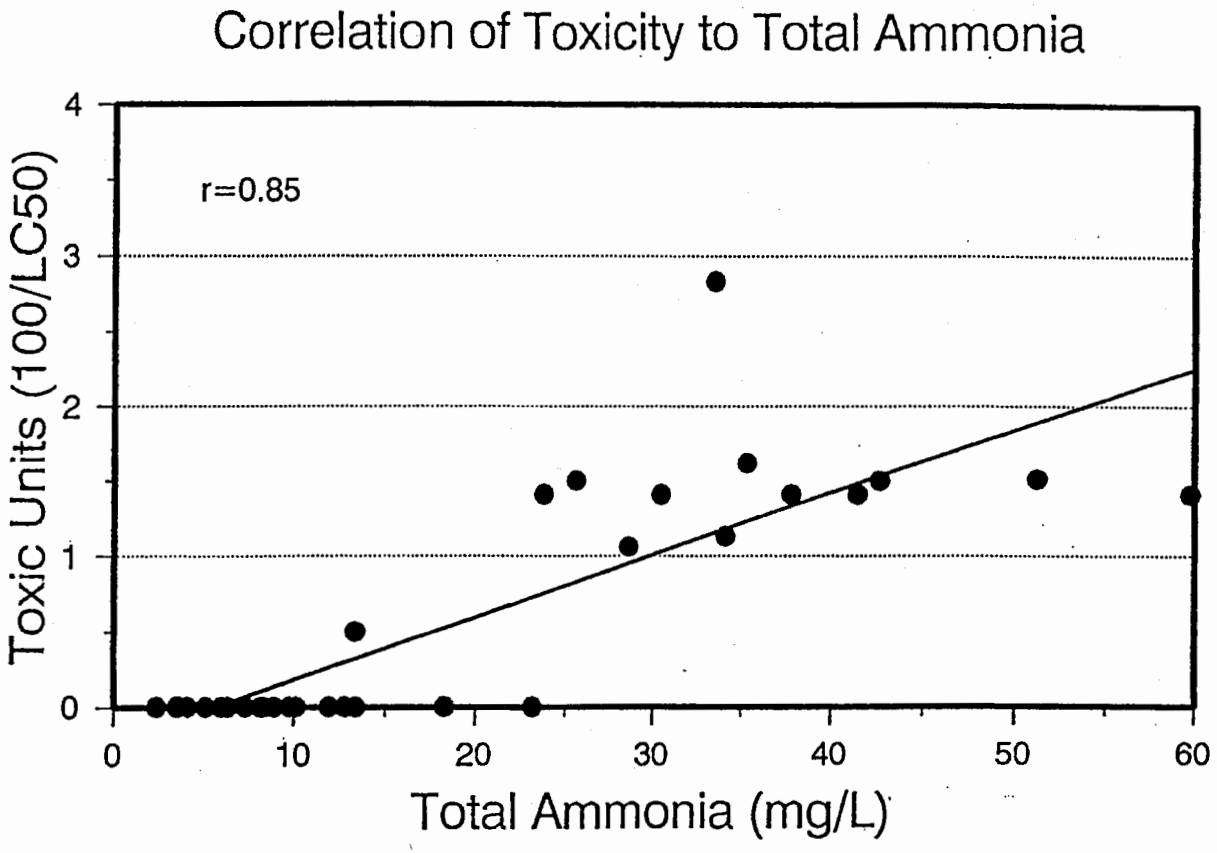


Figure 3.5. Correlation of toxicity with total ammonia concentrations (mg/l ammonia as nitrogen) in sediment porewaters. Toxicity is expressed in toxic units, 100/LC50, where LC50 is the dilution of porewater in clean water that causes half of the Ceriodaphnia dubia to die within 48 hours.

Table 3.1. Characteristics of surface water (S) and sediment pore water (P) from sites on the Illinois Waterway and the Upper Mississippi River.

Station No. (River Mile)		Temp °C	D.O. mg/L	pH	Cond. lmhos/cm	Hardness mg $CaCO_3$/L	Alk. mg $CaCO_3$/L	Total NH_3-N mg/L	Un-ionized NH_3-N mg/L	Chlorine mg/L	H_2S mg/L	S^{2-} mg/l	TOC %
CR326.4	P	23.6	3.20	7.17	1395	488	568	42.0	0.3167	0.10	0.000	0.020	7.15
	S	24.6	9.00	7.28	859	---	---	2.5	0.0260	----	-----	-----	----
CR324.8	P	23.1	4.80	7.08	1071	270	402	28.0	0.1659	0.10	0.000	0.010	6.19
	S	23.0	9.10	7.43	661	---	---	1.6	0.0209	----	-----	-----	----
CS318.5	P	23.7	6.20	7.13	1533	684	422	11.0	0.0762	0.05	0.000	0.020	6.27
	S	20.6	8.20	7.27	1071	---	---	4.0	0.0306	----	-----	-----	----
CS307.4	P	24.0	5.80	6.53	1510	540	506	41.0	0.0733	0.08	0.000	0.020	4.03
	S	22.7	7.20	6.97	571	---	---	1.9	0.0085	----	-----	-----	----
SS317.0	P	22.5	5.80	7.22	798	312	304	15.0	0.1173	0.03	0.000	0.015	7.12
	S	23.1	7.50	7.15	450	---	---	1.6	0.0111	----	-----	-----	----
SS315.3	P	21.0	1.90	6.79	631	254	208	7.3	0.0191	0.08	0.000	0.050	3.23
	S	20.7	8.30	7.13	469	---	---	0.8	0.0045	----	-----	-----	----
SS313.0	P	24.2	0.70	6.85	922	211	336	19.0	0.0718	0.02	0.000	0.070	4.33
	S	26.0	7.60	7.16	532	---	---	0.6	0.0052	----	-----	-----	----
SS310.0	P	27.0	4.60	7.04	740	258	216	11.0	0.0782	0.00	0.000	0.050	0.93
	S	26.5	8.40	6.82	686	---	---	2.2	0.0091	----	-----	-----	----
SS292.2	P	19.9	3.40	7.32	1172	384	390	27.5	0.2241	0.05	0.000	0.070	6.77
	S	22.1	9.40	7.32	1006	---	---	4.6	0.0439	----	-----	-----	----
DP286.3	P	23.3	3.20	7.06	1262	484	478	25.0	0.1435	----	-----	0.080	9.42
	S	26.7	8.60	7.56	1180	---	---	1.9	0.0431	----	-----	-----	----
DP281.1	P	22.0	8.00	7.20	891	468	412	6.7	0.0483	0.05	0.000	0.038	7.63
	S	23.2	9.60	7.70	608	---	---	0.9	0.0220	----	-----	-----	----
DP277.0	P	25.1	7.40	7.84	1366	646	580	10.8	0.4108	0.27	0.000	0.270	3.91
	S	25.4	9.00	8.18	671	---	---	0.6	0.0487	----	-----	-----	----
IR248.2	P	26.1	4.20	7.19	977	382	394	9.5	0.0894	0.02	0.000	0.040	3.17
	S	25.1	8.00	7.59	424	---	---	2.0	0.0435	----	-----	-----	----
IR215.0	P	21.5	5.60	7.48	674	306	257	5.2	0.0685	0.025	0.000	0.030	3.13
	S	20.9	7.90	7.31	537	---	---	1.2	0.0103	----	-----	-----	----

Table 3.1. cont.

Station No. (River Mile)		Temp °C	D.O. mg/L	pH	Cond. µmhos/cm	Hardness mg $CaCO_3$/L	Alk. mg $CaCO_3$/L	Total NH_3-N mg/L	Un-ionized NH_3-N mg/L	Chlorine mg/L	H_2S mg/L	S^{2-} mg/l	TOC %
IR180.0	P	21.4	6.40	7.55	826	346	344	8.3	0.1272	----	-----	0.040	2.34
	S	21.2	8.80	7.55	550	---	---	1.5	0.0227	0.05	0.000	-----	----
IR125.5	P	22.7	6.90	7.41	540	292	278	4.2	0.0514	0.00	0.000	0.180	2.90
	S	21.5	9.30	7.05	559	---	---	0.9	0.0044	----	-----	-----	----
IR 72.0	P	22.3	7.20	7.34	682	352	318	2.0	0.0203	0.15	0.000	0.010	2.39
	S	20.3	9.40	7.63	498	---	---	0.4	0.0068	----	-----	-----	----
IR 6.0	P	25.0	4.80	7.05	757	272	230	3.0	0.0190	0.00	0.000	0.150	0.75
	S	19.6	----	7.84	553	---	---	0.5	0.0130	----	-----	-----	----
MR377.0	P	27.9	5.00	7.10	1011	500	460	14.0	0.1215	0.00	0.000	0.064	1.83
	S	25.7	----	7.19	354	---	---	0.8	0.0073	----	-----	-----	----

Note: 1. mg/l is the same as ppm
2. To convert temperature in °C to °F, multiply by 9/5 and add 32.

Table 3.2. Comparison of responses of fingernail clams[a] (*Musculium transversum*) and water fleas[b] (*Ceriodaphnia dubia*) to sediment pore water from the Des Plaines River portion of the Illinois Waterway (DP277.0). The pore water was subjected to various treatments to remove or change the toxicity of suspected contaminants.

Treatment	pH 3	pH Initial	pH 11
1. Standing			
C. dubia	6.2	13.8[c]	5.6
M. transversum	-0.139	-0.328	-0.202
2. Aerated			
C. dubia	9.9	11.3	6.2
M. transversum	-0.045	-0.431	-0.187
3. Filtered			
C. dubia	0	2.8	1.12
M. transversum	-0.457	-0.795	-0.352
4. C_{18}			
C. dubia	0	1.59	1.4
M. transversum	-0.943	-0.198	-0.328
	pH	pH	pH
5. Graduated pH	6.5	7.5	8.5
C. dubia	Toxic	Toxic	Toxic
M. transversum	-0.513	-0.353	-0.405
		pH	
6. EDTA		Initial	
C. dubia		Toxic	
M. transversum		-0.636	

Notes: [a]The *M. transversum* response is the decline in filtering rate, following 1 hour of exposure to porewater, relative to the control filtering rate. -1.000 = complete cessation of filtering. 0.000 = no reduction.

[b]The *C. dubia* response is measured in toxic units = 100/(48-hour LC50). The LC50 is the percent dilution of porewater (or treated porewater) that kills 50% of the water fleas in 48 hours.

[c]The baseline toxicity of the porewater, prior to any treatment: i.e., the porewater is 13.8 times the lethal level for the water flea and depresses the filtering rate of the clam by 32.8%.

In the first 4 treatments the pH of the porewater is adjusted to the values shown and treated. Then the pH is readjusted to the initial value (7.8) and bioassayed. The porewater is allowed to stand, aerated, filtered, or subjected to reverse-phase, solid phase extraction on a C_{18} column.

In treatment 5, the pH is adjusted to the values shown, and bioassayed.

In treatment 6, the porewater is bioassayed following treatment with the chelating agent EDTA to tie up heavy metals.

However, several sites on the Sanitary and Ship Canal (SS 319.3, SS 312.7, and SS 310.0) where we had obtained clams two months previously (July) now contained few or no live clams, but many recently dead shells, indicating that inimical conditions had developed in the interim (Table 3.3). Another indication of stress was the relatively small size of clams in some areas, such as the Sanitary and Ship Canal at Lockport (SS 292.7, Table 3.3). A normal population in the fall of the year should contain some very small newborns (1-2 mm shell length) and some adults up to at least 12 mm in shell length. Also of note was the presence of the European zebra mussel in the Sanitary and Ship Canal (SS 312.7).

We were successful in obtaining sufficient clams from Pool 19 of the Upper Mississippi River, Swan Lake of the Illinois River, and from two of the waterways in Chicago (the Sanitary and Ship Canal and the Calumet Sag Channel) to run the comparative assays, described next.

3.5.2 Comparative Bioassays. Clams from the six sources differed greatly in the baseline filtering rates of the control animals that were not exposed to added ammonia (Table 3.4). The control rates differed markedly among clams taken from different sites within the same locale; e.g., the control rate for clams from Swan Lake site 1 was nearly double that from site 2 (6.69 vs 3.53 mg yeast/mg clam tissue) and the rate from site 1 of the Sanitary and Ship Canal at Lockport was two thirds that from site 2 (4.15 vs. 6.14). Excluding these two values of 3.53 and 4.15 mg yeast/mg clam, the control rates averaged 6.58 $\pm$ standard deviation of 0.54 (range 6.14 - 7.31). The low control rates may indicate the clams had been subject to some kind of stress at those two sites. Since one site is from the upper waterway (Lockport) and the other from the extreme lower portion of the waterway (Swan Lake), these results indicate that populations throughout the waterway may be under stress, at least in certain local microhabitats.

The experimental groups of clams from the various sources were challenged by exposure to added ammonia. We expected that clams that had been chronically exposed to ammonia might have undergone selection for ammonia tolerance and thus exhibit less response to added ammonia. The clams with the lowest control filtering rate (Swan Lake site 2) actually increased their rate in response to added ammonia, but never came within the control range (Table 3.4). The clams from Swan Lake site 1 did not decrease their filtering significantly (the criterion for a significant response is a change greater than 10.6% from the control rate), but the highest un-ionized ammonia nitrogen concentration achieved in this test (0.0858 mg/l) was lower than the highest concentrations in all of the other tests (0.0943 - 0.2517 mg/l, Table 3.4).

The filtering rate of clams from the Calumet Sag Channel near Chicago increased slightly, but the increase in just one concentration exceeded the response criterion (maximum change was +10.7%). Clams from all other sources decreased their filtering rates significantly when challenged with ammonia. In summary, clams from Swan Lake site 2 appeared to have been under some previous stress (based on their exceptionally low baseline filtering rate) and to be relatively insensitive to ammonia, based on the increase in their filtering rates above baseline in response to added ammonia. Clams from Swan Lake site 1 and from the Calumet Sag Channel likewise showed little response to added ammonia, whereas clams from both sites at Lockport (in the Sanitary and Ship Canal) and from Pool 19 of the Upper Mississippi River

Table 3.3. Qualitative observations on the status of clam populations in Chicago waterways, Pool 19 of the Upper Mississippi River, and Swan Lake of the Illinois River in October and November 1991. Duration of sampling or number of samples is noted.

Site, Date	Qualitative Observations (Mt = *Musculium transversum* Ss = *Sphaerium striatinum*)
MR 365.5 Pool 19, Miss. R. 12 mm. 11 Oct 1991	300 Mt in 2 hr, ranging from newborns to Most were 5-7 mm.
CR 326.4 N. Branch, Chicago R. 29 Oct 1991	150-200 Ss in 2 Ekman grab samples.
CS 318.5 Cal-Sag Channel 29 Oct 1991	200-250 Mt in 4 Ekman samples.
SS 319.3 SS Canal 4 Oct 1991	No live clams in several hours. Substrate consisted mostly of dead clam shells, 10-15% recently dead. Live clams had been obtained in July.
SS 312.7 SS Canal 4 Oct 1991	No native clams, just 15 small zebra mussels in 15 min.
SS 310.0 SS Canal 4 Oct 1991	No live clams.
SS 292.7 SS Canal, Lockport 4 Oct 1991	25 Mt and 25 Ss in about 50 min. No Mt larger than 7 mm. Ss were 6-10 mm.
SS 292.7 SS Canal, Lockport 12 Nov 1991	300 Mt and 75-100 Ss in several hours.
IR 5.1 Swan Lake, Il. R. 9 mm, 17 Oct 1991	325 Mt in 2 hr. 20-30% under 5 mm, 50% 7-10-15% 9-11 mm, 10-15% 11-14 mm.

Table 3.4 Response of fingernail clams (*Musculium transversum*) from different sources to ammonia.

Source of Clams	NH_3-N (mg/l)[a] Initial	Final	Mean	Filtering Rate mg yeast/mg clam[b]/h	Percent Change from Control
Pool 19	0.0023	0.0205	0.0114	7.31	Control
MR 365.5	0.0122	0.0367	0.0245	5.20	-28.9%
	0.0230	0.0275	0.0253	2.52	-65.5%
	0.1168	0.1465	0.1317	1.95	-73.3%
Blue Island	0.0008	0.0037	0.0023	6.19	Control
CS 318.5	0.0152	0.0158	0.0155	6.52	5.3%
	0.0233	0.0180	0.0207	6.85	10.7%
	0.1312	0.0573	0.0943	6.78	9.5%
Lockport	0.0015	0.0039	0.0027	4.15	Control
SS 292.7	0.0132	0.0196	0.0164	2.10	-49.4%
Site 1	0.0188	0.0239	0.0214	1.67	-59.8%
	0.0991	0.1420	0.1206	1.63	-60.7%
Lockport	0.0016	0.0021	0.0019	6.14	Control
SS 292.7	0.0279	0.0402	0.0341	5.92	-3.6%
Site 2	0.0625	0.0702	0.0664	3.36	-45.3%
	0.2535	0.2499	0.2517	1.34	-78.2%
Swan Lake	0.0028	0.0053	0.0041	6.69	Control
IR 5.1	0.0106	0.0252	0.0179	6.18	-7.6%
Site 1	0.0195	0.0256	0.0226	6.68	-0.1%
	0.0955	0.0760	0.0858	6.00	-10.3%
Swan Lake	0.0032	0.0035	0.0034	3.53	Control
IR 5.1	0.0177	0.0213	0.0195	4.66	32.0%
Site 2	0.0256	0.0305	0.0281	5.84	65.4%
	0.0850	0.1045	0.0948	5.86	66.0%

[a] Concentration is expressed as un-ionized ammonia nitrogen
[b] Dry tissue weight, minus shell

decreased their filtering rates substantially in response to ammonia. Clams from different sources do vary in their tolerance of ammonia, but their tolerance does not increase in the upstream direction on the Illinois Waterway, where ammonia concentrations in sediment pore water tend to be higher than in downstream areas.

4.0 DISCUSSION

4.1 The Fingernail Clam Filtering Bioassay

The fingernail clam filtering bioassay can be performed with relatively simple equipment commonly available in most laboratories. It is relatively quick, requiring only one hour of exposure to the test solution, followed by one hour to assess filtering performance. It measures a sublethal response, filtering ability, that is critical to the survival of the organism, so the bioassay meets the test of physiological relevance to the organism. The bioassay also meets the test of ecological relevance because the test species, *M. transversum*, is an important link in food chains leading to fish and ducks and it is a dominant member of the benthic macroinvertebrate community in many Midwestern aquatic systems. Another aspect of ecological relevance is that *M. transversum* is a member of the infauna, the organisms that burrow into the sediment rather than living on top of the sediment or on plants, snags or other structure extending into the water column, so it is actually exposed in the environment to the sediment porewater we were testing. In contrast, many of the standard test organisms, including *C. dubia*, are water column dwellers or structure dwellers that never come in contact with sediment porewater in nature. The filtering response is graded; i.e., the degree of inhibition of filtering performance is a function of the concentration of the toxicant, as demonstrated in the test with a reference toxicant, sodium cyanide.

The results obtained by the two types of clam bioassays were generally consistent. In one bioassay, the beating rate of cilia on isolated clam gills was inhibited by bulk sediments from the upper Illinois Waterway, even though the sediments were diluted with molluscan saline solution. In the other bioassay, the filtering rate of intact clams was inhibited by sediment porewaters from the same area. The same porewaters caused significant mortality in *C. dubia*. In contrast, porewaters from some of the same sites stimulated two other standard test organisms, the freshwater alga *Selenastrum capricornutum* and the marine bacterium *Photobacterium phosphoreum* (Microtox™ test). In view of the great physiological differences among plants, bacteria and animals this result is not too surprising; e.g., ammonia is toxic to aquatic animals at relatively low concentrations, but is used as a nitrogen source by plants and some bacteria.

4.2 Toxicity in the Illinois Waterway

Two different patterns of toxicity apparently occur in the sediment porewaters of the Illinois Waterway. There is a gradient of increasing toxicity in the upstream direction, associated with increasing concentrations of total ammonia in the sediments (Figures 3.2, 3.4, and 3.5). The second pattern is characterized by patches of toxicity associated with polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, and long-chain hydrocarbons, both evidently derived from petroleum (Sparks, Ross and Dillon 1992). One of the latter sites was located on the lower Des Plaines River section of the waterway, near several refineries. Previous studies have measured elevated levels of metals, pesticides, PAHs, and PCBs in the sediments of the upper

Illinois Waterway (IEPA 1990) and demonstrated that sediments are toxic (Sparks, Sandusky and Paparo 1981; Blodgett et al. 1984; Schubauer-Berigan and Ankley 1991). The two toxicity problems might even be related: Ankley et. al. (1991) suggest that natural microbial processes in aquatic ecosystems may be compromised by organic loading or selective toxicity. The alteration of microbial processes could play a role in the incidence of ammonia accumulation and subsequent toxicity in sediments in the Upper Illinois Waterway.

It is well established that certain sediments can contain high concentrations of ammonia (Keeney 1973, Berner 1980). Nitrogen-containing organic matter is decomposed in sediments by heterotrophic bacteria. The amount of ammonification that takes place depends on oxygen availability (Kleerekoper 1953). Ammonia can accumulate to toxic levels under anaerobic conditions (Berner 1980). Ammonia formation is greatest about 10 cm below the sediment-water interface (Serruya 1974). In this situation, ammonia probably diffuses from the deeper sediments to surficial sediments, and perhaps even to the overlying water, especially if sediments are resuspended by currents or boat- or wind-driven waves. The fingernail clam, *Musculium transversum*, the organism of primary interest in this study, makes shallow burrows in the sediment and may be exposed to much higher levels of ammonia than organisms living in the water column.

M. transversum is sensitive to ammonia. Anderson, Sparks and Paparo (1978) found that un-ionized ammonia concentrations of 0.08-0.09 mg/l inhibited the cilia on the gills of the clams, and the growth of the clams in the laboratory was reduced at concentrations between 0.20 and 0.34 mg/l NH_3-N. The *C. dubia* acute LC50 for ammonia is 1.04 mg/l (Ankley et. al. 1990). Arthur et. al (1987) reported un-ionized ammonia toxicity to 5 invertebrates that ranged from 1.95 to 18.3 mg/l un-ionized ammonia, as nitrogen (see below).

SPECIES	LC50 (mg/l)
Snail	
Physa gyrina - adult	1.95
Helisoma trivolvis - adult	2.17
Amphipod	
Crangonyx pseudogracilis - adult	3.12
Mayfly	
Callibaetis skokianus - nymph	3.12
Isopod	
Asellus racovitzai - adult	5.02
Caddisfly	
Philarctus giaeris - larvae	10.1
Crayfish	
Orconectes immunis - adult	18.3

Concentrations of un-ionized NH_3-N ranged up to 0.41 in the sediments in the Upper Illinois Waterway, based on total ammonia concentrations and pHs measured in samples returned to the laboratory (Table 3.1)--sufficient to impair filtering and reduce growth. Ammonia places organisms in double jeopardy because it exerts an oxygen demand in the process of nitrification (conversion to nitrites and then nitrates) and low oxygen levels place organisms under additional stress (USEPA 1985). Ammonification may be occurring in the deep, anaerobic zones of the sediments and nitrification in the shallower, aerobic zones, or in the boundary water at the sediment surface, so benthic invertebrates are exposed to the worst of both worlds: they are exposed to ammonia and to low oxygen at the same time.

The highest ammonia concentrations in sediments are associated with nitrogen-enriched sediments or high organic loading, as from sewage treatment plants (Brezonik 1973; Ankley et. al. 1990; and Schubauer-Berigan and Ankley 1991). Although most sewage treatment plants remove a substantial portion of carbon that is in municipal waste, most do not remove nitrogen, but convert it from ammonia into nitrate. It is possible that nitrate is carried down into the sediments where it is converted back into ammonia in the anaerobic zones. If this is the case, ammonia toxicity in the sediments might be reduced by reducing the nitrogen loading of the river.

The proportion of total ammonia existing in the toxic, un-ionized form is controlled primarily by pH and temperature (Emerson et. al. 1975). The pH of sediments can fluctuate dramatically on a seasonal basis, and the pH of the overlying water can fluctuate daily, so that episodes of toxicity may occur even if the total ammonia concentration remains relatively constant. Ammonia loading of rivers tends to increase during winter because the microorganism-mediated conversion of ammonia to nitrate stops at cold temperatures. Also, aquatic vegetation does not remove ammonia (a plant nutrient) during winter dormancy. Water quality standards frequently allow higher levels of ammonia in the winter because the proportion of total ammonia existing in the toxic, un-ionized form is less at cold temperatures. However, the sensitivity of fish to ammonia increases at cold temperatures, and this biological effect overrides the physical-chemical effect of cold temperature on ammonia equilibria (Reinbold and Pescitelli 1990). Research is needed to determine the effect of cold temperatures on the sensitivity of invertebrates, such as fingernail clams, as well as fish, to ammonia.

During the course of this study, several species of fingernail clams, including *M. transversum*, reappeared in the Chicago area waterways and in the Illinois River at Peoria and Havana. There are at least four possible explanations for this surprising reappearance of clams in the same general areas where the porewaters tested toxic. First, we found that clams from different locations differ in their sensitivity to ammonia, and clams from at least one site on the upper Illinois (the Calumet Sag Channel) responded less to added ammonia than the clams from Pool 19 and from one site in Swan Lake on the lower Illinois, where the organisms were obtained for all of the early bioassays. Second, our previous research demonstrated that the surface layers of sediment in some areas are less toxic than layers a few centimeters deeper (Sparks, Sandusky and Paparo 1981; Blodgett et al. 1984). Toxicity may have been overestimated in tests where surface and deep layers of sediment were mixed prior to testing. Third, toxic

episodes may be brief and infrequent, allowing organisms to colonize in between episodes. In the span of just two months, we sometimes found live clams in the Sanitary and Ship Canal where there had been none before, and conversely, recently-dead shells where live clams had been relatively abundant (see section 3.5.1 and Table 3.3). Fourth, the distribution of toxicity in sediments may be extremely patchy, so that healthy organisms are found adjacent to barren or stressed areas. Clams from adjacent sites in both Swan Lake on the lower Illinois River and at Lockport in the Chicago Sanitary and Ship Canal differed markedly in their control filtering rates and in their sensitivity to added ammonia, indicating that they had experienced different levels of stress prior to collection (Table 3.4). If the latter two hypotheses prove to be true, toxicity in the Illinois River has changed recently from a widespread, chronic problem to a more localized or episodic problem. Reduction of toxicity in surface sediments may reflect recent reductions in ammonia loading from sewage treatment plants in the Chicago area, although it is not clear whether the sources of ammonia in the porewaters are effluents, the deeper layers of sediments (as described above), or both.

We remind the reader that all the toxicity tests we conducted were short-term, acute tests. The fingernail clams, *Musculium transversum*, were exposed to sediment porewater for only 1 hour and then their filtering performance was tested in clean dilution water. The water flea, *Ceriodaphnia dubia*, was exposed to porewater for just 48 hours. The organisms in the waterways are exposed to contaminants for their entire life spans. In the past, more sensitive tests with fingernail clams have demonstrated toxicity even in downriver sediments, including fish and wildlife areas such as Peoria Lake and Quiver Lake (Sparks, Sandusky and Paparo 1981).

In addition to being a problem for the benthic invertebrates that fish feed upon, ammonia may be a problem for the fish themselves. In 1987, the U.S. Fish and Wildlife Service simulated resuspension of bottom sediments by boat- or wind-driven waves by stirring sediments in clean water, allowing the sediment to settle for 24 to 48 hours, then exposing larval fathead minnows, *Pimephales promelas*, to the water. Water mixed with surface sediments from the Chicago River and the Des Plaines River killed all the fish within 24 hours. Surface sediments from Lake Chautauqua, a bottomland lake and federal wildlife refuge along the Illinois River at Havana, killed 15% of the test fish in 96 hours; deeper sediments, taken at the 12- to 18-inch depth, killed 25%. Fish mortality correlated ($R = 0.71$, $P < 0.01$) with the concentration of un-ionized ammonia released from the sediment and both ammonia and fish mortality increased upstream toward Chicago. The Long-Term Research Monitoring Station (LTRM) at Havana started measuring ammonia concentrations in Anderson Lake, a floodplain lake of the Illinois River and a state fish and wildlife area, on 1 May 1990, 2 days after a fish kill. The total ammonia nitrogen concentration was 0.90 mg/l and the un-ionized ammonia nitrogen was calculated to be 0.36 mg/l at the temperature of 16.6^{0} C and pH of 9.34. NH_3-N concentrations of 0.32 mg/l at 3-5^{0} C and 1.35 mg/l at 24-25^{0} C were acutely lethal to bluegill sunfish in bioassays conducted at our laboratory. The fish kill might have been caused by ammonia, if the un-ionized ammonia had peaked at higher concentrations before our samples were taken.

Elevated un-ionized ammonia concentrations might be triggered by resuspension of sediments or episodes of elevated pH resulting from

phytoplankton blooms. Plants remove carbon dioxide from the water, in the form of carbonic acid and bicarbonate, and thereby elevate the pH of the water, which in turn increases the proportion of ammonia existing the toxic, un-ionized form. The Havana LTRM station measured pHs as high as 10.12 in backwater lakes of the Illinois River in July 1990 and values between 9.0 and 10.0 occur fairly often. Episodes of acute ammonia toxicity thus may be occurring sporadically in places other than just the upper Illinois River, and it takes only one brief episode per year to kill or reduce populations that take many months or years to build up. Potential sources of ammonia or nitrogen, besides sewage plants and anaerobic sediments, include industrial plants (especially refineries and munitions plants), feedlots, and agricultural fields.

Although a general recovery does seem to be beginning in the Illinois River, with the return of fingernail clams in some areas where they have been absent at least 30 years and appearance of largemouth bass throughout the Illinois River proper, the pace and permanence of recovery still appears to be threatened by ammonia, even if the problem now turns out to be episodic instead of chronic. Reports of fingernail clam and mussel die-offs in the Upper Mississippi River and other rivers (Wilson et al. in press; Blodgett and Sparks 1987; Neves 1987) indicate that drastic population declines in macroinvertebrates that burrow in sediments are not unique to the Illinois River.

5.0 LITERATURE CITED

Adams, W.J., R.A. Kimerle, and R.G. Mosher. 1985. Aquatic safety assessment of chemicals sorbed to sediments. Pages 429-453 *in* R.D. Cardwell, R. Purdy, and R.C. Bahner, eds. Aquatic Toxicity and Hazard Assessment: Seventh Symposium, ASTM 854, American Society for Testing and Materials, Philadelphia, PA.

Aldridge, D.W., B.S. Payne, and A.C. Miller. 1987. The effects of intermittent exposure to suspended solids and turbulence on three species of freshwater mussels. Environmental Pollution 45:17-28.

American Public Health Association (APHA). 1976. Standard methods for the examination of water and wastewater. 14th Edition. American Public Health Association, Washington, D.C. 1193 p.

Anderson, K.B., R.E. Sparks, and A.A. Paparo. 1978. Rapid assessment of water quality using the fingernail clam, *Musculium transversum*. University of Illinois Water Resources Center, Center Research Report No. 133.

Anderson, R.V., D.M. Day, M. Demissie, F.S. Dillon, J.W. Grubaugh, M.S. Henebry, K.S. Lubinski, and R.E. Sparks. 1984. Flows, equations and input values for the nine state-vaiable biological model, Pool 19, Mississippi River--second generation. A computer-modelling project of the Large-River, Long-Term Ecological Research Project (LTER). Unpublished report. 106 pp.

Ankley, G.T., A. Katko, and J. Arthur. 1990. Identification of ammonia as an important sediment-associated toxicant in the lower Fox River and Green Bay, Wisconsin. Environmental Toxicology and Chemistry 9:313-322.

Ankley, G.T., K. Lodge, D.J. Call, M.D. Balcer, L.T. Brooke, P.M. Cook, R.G. Kreis, Jr., A.R. Carlson, R.D. Johnson, G.J. Niemi, R.A. Hoke, C.W. West, J. P. Giesy, P.D. Jones, and Z.C. Fuying. 1991. Integrated assessment of contaminated sediments in the lower Fox River and Green Bay, Wisconsin. Ecotoxicology and Environmental Safety 23:46-63.

Arthur, J.W., W.W. Corlis, K.N. Allen, and S.F. Hedtke. 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. Bulletin of Environmental Contamination and Toxicology 38:324-331.

ASTM. 1982. Annual book of ASTM standards. Part 31. Water. American Society for Testing and Materials, Philadelphia, PA. 1554 pp.

ASTM. 1991. Annual book of ASTM standards. Water. American Society for Testing and Materials, Philadelphia, PA.

Berner, R.A. 1980. Early Diagenesis: a theoretical approach. Princeton University Press, Princeton, NJ.

Blodgett, K.D. 1983. Distribution of toxicity in sediments of the upper Illinois Waterway. M.S. Thesis. Western Illinois University, Macomb. 72 p.

Blodgett, K.D., R.E. Sparks, A.A. Paparo, R.A. Cahill, and R.V. Anderson. 1984. Distribution of toxicity in sediments of the Illinois Waterway. Pages 293-308 *in* W. Blake-Coleman, ed. Proceedings of the Conference of Urban Effects on Water Quality and Quantity. Urbana, IL 20-21 October 1983.

Blodgett, K.D., and R.E. Sparks. 1987. Documentation of a mussel die-off in Pools 14 and 15 of the Upper Mississippi River. Pages 76-90 *in* R.J. Neves, ed. Proceedings of the workshop on Die-Offs of Freshwater Mussels in the United States. U.S. Fish and Wildlife Service and Upper Mississippi River Conservation Committee. 23-25 June, Davenport, IA.

Brezonik, P.L. 1973. Nitrogen sources and cycling in natural waters U.S. Environmental Protection Agency, National Environmental Research Center, Washington, D.C. EPA60013-73-002. 167 pp.

Bulich, A.A., M.W. Greene, and D.L. Isenberg. 1981. Reliability of the bacterial luminescence assay for the determination of the toxicity of pure compounds and complex effluents. Pages 338-347 *in* ASTM-STP No. 737, Philadelphia.

Connell, D.W., M. Bowman, and D.W. Hawker. 1988. Bioaccumulation of chlorinated hydrocarbons from sediment by oligochaetes. Ecotoxicology and Environmental Safety 16:293-302.

Di Toro, D.M., J.D. Mahony, D.J. Hansen, L.J. Scott, A.R. Carlson, and G.T. Ankley. 1992. Acid volatile sulfide predicts the acute toxicity of cadmium and nickel in sediments. Environmental Science and Technology 26(1):96-101.

Eckblad, J.W. 1978. Laboratory manual of aquatic biology. Wm. C. Brown Company Publisher, Dubuque, IA. 231 p.

Edmunds, W.M., and A.H. Bath. 1976. Centrifuge extraction and chemical analysis of intersitial waters. Environmental Science and Technology 10:467-472.

Emerson, K., R.C. Russo, R.E. Lund, and R.V. Thurston. 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. Journal of Fisheries Research Board of Canada 32:2379-2383.

Emmling, Philip J. 1974. Sphaeriid-sediment interaction in the Milwaukee Harbor, Lake Michigan. Page 11 *in* Midwest Benthological Society titles & abstracts of papers presented at the 22nd Annual Meeting, March 27-29, NERC, Cincinnati, OH.

Gale, W.F. 1969. Bottom fauna of Pool 19, Mississippi River with emphasis on the life history of the fingernail clam, *Sphaerium transversum*. Ph Dissertation Iowa State University of Science and Technology, Ames. 235 p.

HACH. 1985. HACH water analysis handbook. HACH Company. 359 p.

Hamilton, M.A., R.C. Russo, and R.B. Thurston. 1977. The Trimmed Spearman Carver method for estimating median lethal concentrations in toxicity bioassays. Environmental Science and Technology 11:714-719.

Howes, B.L., J.W.H. Dacey, and J.M. Teal. 1985. Annual carbon mineralization and belowground production of *Spartina alterniflora* in a New England salt marsh. Ecology 66(2):595-605.

Hubert, W.A. 1972. Seasonal fluctuations of aquatic macroinvertebrates in Oakwood Bottoms Greentree Reservoir. B.S. Thesis. Southern Illinois University. 19 p.

Illinois Environmental Protection Agency. 1990. Illinois water quality report 1988-1989. IEPA/WPC/90-160. Illinois Environmental Protection Agency, Division of Water Pollution Control, Springfield, IL. 352 pp.

Jude, D.J. 1968. Bottom fauna utilization and distribution of 10 species of fish in Pool 19, Mississippi River. M.S. Thesis. Iowa State University, Ames. Unpublished. 238 p.

Jude, D.J. 1973. Food and feeding habits of gizzard shad in Pool 19, Mississippi River. Transactions of the American Fisheries Society 102 (2):378-383.

Keeney, D.R. 1973. The nitrogen cycle in sediment-watersystems. Journal of Environmental Quality 2:15-29.

Kleerekoper, H. 1953. The mineralization of plankton. Journal of Fisheries Research Board of Canada 10:283-291.

Knezovich, J.P., and F.L. Harrison. 1988. The bioavailability of sediment-sorbed chlorobenzenes to larvae of the midge, *Chironomus decorus*. Ecotoxicology and Environmental Safety 15:226-241.

Marking, L.L., and V.K. Dawson. 1973. Toxicity of quinaldine sulfate to fish. Invest. Fish Contr. No. 48, U.S. Fish and Wildlife Service. Washington, D.C. 8 p.

Mount, D.I. 1988. Methods of aquatic toxicity identification evaluations: phase III toxicity confirmation procedures. EPA Research Series Report. Environmental Research Laboratory, Duluth, MN. EPA/600/3-88/036.

Mount, D.I., and L. Anderson-Carnahan. 1988. Methods of aquatic toxicity identification evaluations: phase I toxicity characterization procedures. EPA Research Series Report. Environmental Research Laboratory, Duluth, MN. EPA/600/3-88/034.

Mount, D.I., and L. Anderson-Carnahan. 1989. Methods of aquatic toxicity identification evaluations: phase II toxicity identification procedures. EPA Research Series Report. Environmental Research Laboratory, Duluth, MN. EPA/600/3-88/035.

National Effluent Toxicity Assessment Center (NETAC). 1989a. Standard operating procedures for *Ceriodaphnia* culturing. 4 p.

National Effluent Toxicity Assessment Center (NETAC). 1989b. Standard operating procedures for *Ceriodaphnia* food regime. 4 p.

Neves, R.J., ed. 1987. Proceedings of the workshop on die-offs of freshwater mussels in the United States. U.S. Fish and Wildlife Service and Upper Mississippi River Conservation Committee. 23-25 June, Davenport, IA.

Pennak, R.W. 1989. Fresh-water invertebrates of the United States: Protozoa to Mollusca. John Wiley & Sons Inc., New York. 628 p.

Peterson, A. 1951. Larvae of insects: an introduction to Nearctic species: Coleoptera, Diptera, Neuroptera, Siphonaptera, Mecoptera, Trichoptera Part II. Edward Brothers Inc., Ann Arbor, MI. 416 p.

Paloumpis, A.A., and W.C. Starrett. 1960. An ecological study of benthic organisms in three Illinois River flood plain lakes. American Midland Naturalist 64(2):406-435.

Ranthum, Richard Gilbert. 1969. Distribution and food habits of several species of fish in Pool 19, Mississippi River. M.S. Thesis, Iowa State University, Ames, IA. 207 p.

Ross, P.E., V. Jarry, and H. Sloterdijk. 1988. A rapid bioassay using the green alga, *Selenastrum capricornutum*, to screen for toxicity in St. Lawrence River sediment elutriates. Pages 68-73 *in* J. Cairns, Jr., and J.R. Pratt, eds. Functional Testing of Aquatic Biota for Estimating Hazards of Chemicals. American Society for Testing Materials, Special Technical Publication No. 988, Philadelphia, PA. 242 pp.

Schubauer-Berigan, M.K., and G.T. Ankley. 1991. The contribution of ammonia, metals and nonpolar organic compounds to the toxicity of sediment interstitial water from an Illinois River tributary. Environmental Toxicology and Chemistry 10:925-939.

Serruya, C. 1974. Nitrogen and phosphorus balances and load-biomass relationship in Lake Kinneret. Verh. Internat. Verein. Limnol. 19:1357-1369.

Snell, T.W., and G. Personne. 1989. Acute toxicity bioassay using rotifers II. A freshwater test with *Brachionus rubens*. Aquatic Toxicology 14:81-92.

Sparks, R.E. 1984. The role of contaminants in the decline of the Illinois River: Implications for the Upper Mississippi. Pages 25-66 *in* J.G. Wiener, R.V. Anderson, and D.R. McConville, eds. Contaminants in the Upper Mississippi River. Proceeceedings of the 15th Annual Meeting of the Mississippi River Research Consortium. Butterworth Publishers, Stoneham, MA. 368 pp.

Sparks, R.E., M.J. Sandusky, and A.A. Paparo. 1981. Identification of the water quality factors which prevent fingernail clams from recolonizing the Illinois River, Phase II. University of Illinois, Water Resources Center Research Report No. 157. Urbana, IL.

Sparks, R.E., and K.D. Blodgett. 1983. Toxicity of sediments in the Illinois Waterway: implications for the proposed increases in diversion of Lake Michigan water into the Illinois Waterway. Project Completion Report STIL-TRWRTOXSED39483, U.S. Army Corps of Engineers, Chicago, IL.

Sparks, R.E., and M.J. Sandusky. 1983. Identification of the water quality factors which prevent fingernail clams from recolonizing the Illinois River, Phase III. University of Illinois Water Resources Center Research Report No. 179. Urbana, IL. 55 pp.

Sparks, R.E., M.J. Sandusky, and A.A. Paparo. 1983. Identification of the water quality factors which prevent fingernail clams from recolonizing the Illinois River, Phase III. Water Resources Center Research Report No. 179, University of Illinois, Champaign-Urbana. 53 p.

Sparks, R.E., P.E. Ross, and F.S. Dillon. 1992. Identification of toxic substances in the upper Illinois River. Illinois Department of Energy and Natural Resources, Springfield. Final Report Contract WR3691. ILENR/RE-WR-92/07. 59 p.

Swartz, R.C., G.R. Ditsworth, D.W. Schults, and J.O. Lamberson. 1985. Sediment toxicity to a marine infaunal amphipod: cadmium and its interaction with sewage sludge. Marine Environmental Research 18:133-153.

Swartz, R.C., P.F. Kemp, D.W. Schults, and J.O. Lamberson. 1988. Effects of mixtures of sediment contaminants on the infaunal amphipod, *Rhepoxynius abronius*. Environment Toxicology and Chemistry 7:1013-1020.

Thompson, C.M., and R.E. Sparks. 1978. Comparative nutritional value of a native fingernail clam and the introduced Asiatic clam. The Journal of Wildlife Management 42(2):391-396.

U.S. Army Corps of Engineers. 1987. Illinois River charts. U.S. Army Corps of Engineers.

U.S. Environmental Protection Agency. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. Environmental Research Laboratory, Cincinnati, OH. EPA/600/4-85-013.

Wilson, D.M., T.J. Naimo, J.G. Wiener, R.V. Anderson, M.B. Sandheinrich, and R.E. Sparks. (Submitted). Declining populations of the fingernail clam *Musculium transversum* in the Upper Mississippi River. Submitted to the Journal of the North American Benthological Society.

Appendix Table A.1. Relationship between yeast concentration and % light transmittance measured by a spectrophotometer.

Yeast mg/l	% Transmittance			
	x1	x2	x3	$\bar{x}$
100	86.50	86.00	86.00	86.17
90	87.10	87.00	86.98	87.02
80	87.80	87.20	87.20	87.40
70	89.00	89.00	89.10	89.03
60	90.13	90.31	90.35	90.26
50	91.69	91.68	91.71	91.69
40	93.25	93.23	93.30	93.26
30	94.70	94.73	94.80	94.74
20	96.61	96.45	96.45	96.50
10	99.22	99.39	99.47	99.36

Yeast concentrations were calculated from a regression equation based on the above data:

Y = yeast concentration (mg/l)

T = % transmittance of light

$$Y = 676.378 - 6.788\ T$$

NEW DOCUMENT

Morphological examination of resting egg structure of three cladoceran species (*Ceriodaphnia quadrangula* (O.F. Müller, 1785), *Daphnia longispina* (O.F. Müller, 1776) and *D. magna* Straus, 1820)

Murat KAYA[1,2,*], Sevil ERDOĞAN[2,3]

[1]Aksaray University, Faculty of Science and Letters, Department of Biotechnology and Molecular Biology, 68100, Aksaray, Turkey; E-Mail: muratkaya3806@yahoo.com

[2] Aksaray University, Science and Technology Application and Research Center, 68100, Aksaray, Turkey.

[3] Trakya University, Keşan Vocational College, Fisheries Programme, 22800, Keşan, Edirne, Turkey; E-mail: serdogantrakya@gmail.com

***Corresponding author: Murat KAYA**

Address: Aksaray University, Faculty of Science and Letters, Department of Biotechnology and Molecular Biology, 68100, Aksaray, Turkey.

E-Mail: muratkaya3806@yahoo.com

Tel.: +90-382-288-2184

Fax: +90-382-288-2125

Abstract

Morphological characteristics of resting egg which is important to ensure the continuity of generations were examined to understand if there are morphologically significant differences between three cladoceran species. Resting eggs floating on water surface were collected by plankton net with mesh size of 100 µm from Mamasın Dam Lake in November 2012 and were left to dry at room temperature in the laboratory. In this study, size, shape and colour of ephippium and size of the egg/eggs enclosed in ephippium were comparatively analysed by stereo microscope and scanning electron microscope (SEM). It was observed each resting egg type has its characteristic shape and colour. Results of the statistical analysis showed there are statistically significant differences between sizes of ephippium and egg/eggs enclosed in ephippium of resting egg of each species. We concluded that morphological features of resting eggs of three species are different from each other and these characters can be used for species identification. In addition, we described morphological features of resting eggs of three cladoceran species in detail, gave extra information about colour and size differences and contributed to improve the literature knowledge of these species with this study.

Key words: SEM, resting egg, size, shape, colour

1. Introduction

Cladoceran species resume their lives by reproducing asexually, with thin-shelled summer eggs under normal circumstances (Davison, 1969; Boersma et al., 2000). In unfavourable environmental conditions (e.g. low water temperature, predation pressure, surviving during the dry period), males appear and thick-shelled, resistant, fertilized eggs (called 'resting egg') are produced (Slusarczyk, 2001; Slusarczyk and Pietrzak, 2008; Panarelli et al., 2008). When conditions return to normal, these resting eggs hatch and create new offsprings; thus organisms ensure the continuity of generations by means of these eggs (Panarelli et al., 2008; Vanickova et al., 2010). It was observed in former studies that resting egg can survive for many decades without decomposing (Caceres, 1998; Rother et al., 2010).

Until now, studies about resting egg generally focused on subjects as genetic characterization of resting egg (Reid et al., 2000), maternal effect on the size of resting egg (Boersma et al., 2000), resistance to predation (Slusarczyk, 2001), ecological and evolutionary significance of resting egg (Brendonck and De Meester, 2003), importance of resting egg in terms of continuity of zooplankton populations (Jankowski and Straile, 2003; Panarelli et al., 2008; Conde-Porcuna et al., 2011), buoyancy of ephippium (Slusarczyk and Pietrzak, 2008) and hatching of resting egg (Rother et al., 2010; Haghparast et al., 2012).

In this study, we comparatively analysed size, shape and colour of resting egg and the egg/eggs enclosed in ephippium of three different cladoceran species (*Ceriodaphnia quadrangula* (O.F.Müller, 1785), *Daphnia longispina* (O.F.Müller, 1776) and *D. magna* Straus, 1820) to understand if there are significant morphological differences between resting eggs of three cladoceran species.

2. Materials and Methods

Following the winter eggs production time of cladoceran species in Mamasın Dam Lake, tonnes of floating resting eggs were observed on water surface in November 2012. Resting eggs were collected by plankton net with mesh size of 100 µm from the surface of water and were left to dry at room temperature in the laboratory. Dry resting eggs were examined under the microscope, and three different types of resting eggs were determined. These three different resting eggs were cultured under the laboratory conditions (at temperature of 28°C and under the fluorescent light of 514 watt) and species hatching from resting eggs were identified according to Benzie (2005). After that, resting eggs were sifted through the sieves with mesh sizes of 200 µm and 400 µm and resting eggs of different species were separated from each other by making use of size differences. One hundred resting eggs from each species were randomly picked, and length and width of each ephippia and eggs enclosed in ephippia were measured under the Leica DM 4000 binocular microscope. In addition, photos of resting eggs were taken with a Leica DFC 295 camera attached to a Leica Z6 APO zoom system at magnification of ×32-36 and the surface morphology of the resting eggs were examined by EVO LS 10 ZEISS scanning electron microscope at magnifications of ×70-200-300. The resting eggs were coated with gold for SEM analysis by Sputter Coater (Cressingto Auto 108).

We applied normality test to check if data followed normal distribution or not. We saw that data does not follow normal distribution. Therefore, we approved to use a non-parametric test. We used Mann-Whitney U test to understand if there is statistically

significant difference between species in terms of sizes of ephippia and eggs enclosed in ephippia. Analysis of data was performed using SPSS (v.16).

3. Results

Identification of the individuals hatched from cultivated resting eggs showed that these resting eggs belong to three different cladoceran species: *Ceriodaphnia quadrangula*, *Daphnia longispina* and *D. magna*.

3.1. Resting egg shape

It was observed that resting eggs of three cladoceran species have different morphological characteristics (Figure 1).

Ephippia of *C. quadrangula* is semicircular, symmetrical and carries floating cells. Egg chamber containing only one egg is located parallel and close to dorsal margin of ephippium. Dorsal margin does not have anterior and posterior projections (Figure 1A and D).

Ephippia of *D. longispina* is in shape of "D" letter narrowing at postero-ventral side. Egg chambers carrying two eggs are perpendicularly located to the spinose dorsal margin of ephippium and ephippium do not have anterior and posterior projections (Figure 1B and E).

Ephippia of *D. magna* is in shape of straight "D" letter. Egg chambers containing two eggs are located more or less horizontally at an angle to the spinose dorsal margin of ephippium. Ephippium is long and carries anterior and posterior projections (Figure 1C and F).

120 **3.2. Resting egg colour**

121 Stereo microscope analysis indicated that the background colour of ephippia of
122 *C. quadrangula* is whitish cream and margins are transparent. Egg is orange brown
123 (Figure 1D).

124 Ephippia of *D. longispina* is covered with a transparent outer cover, background
125 colour of ephippia ranges from white to light brown and egg chambers are deep brown
126 (Figure 1E).

127 Background colour of ephippia of *D. magna* seems cream under stereo
128 microscope, dorsal margin is deep brown or grey and egg chambers are brown (Figure
129 1F).

130

131 **3.3. Resting egg size**

132 As a result of measurements, it was determined that ephippium sizes vary largely
133 according to different species.

134 Ephippia of *D. magna* is approximately three times larger than ephippia of *D.*
135 *longispina* and four times larger than ephippia of *C. quadrangula.* Size difference
136 between ephippia of *D. longispina* and *C. quadrangula* is approximately 100 μm (Table
137 1).

138 Mann-Whitney U test indicated that there is a statistically significant difference
139 between *D. magna* and *D. longispina*, *D. magna* and *C. quadrangula* and *D. longispina*
140 and *C. quadrangula* in terms of ephippia length and width according to minimum
141 $p<0.05$.

142

143 **3.3.1. Sizes of eggs enclosed in ephippium**

144 It was observed that eggs enclosed in ephippia of *D. magna* are quite larger than
145 the other two species. Although, eggs enclosed in ephippia of *D. longispina* are slightly
146 larger than the eggs of *C. quadrangula*, there is almost no difference between their sizes
147 (Table 2).

148 Considering the result of Mann-Whitney, it was revealed that there is
149 statistically significant difference between *D. magna* and *D. longispina*, *D. magna* and
150 *C. quadrangula* and *D. longispina* and *C. quadrangula* in terms of width of eggs
151 enclosed in ephippia according to minimum $p<0.05$. When we considered the length of
152 eggs enclosed in ephippia, we observed although, there is a statistically significant
153 difference between *D. magna* and *D. longispina*, *D. magna* and *C. quadrangula*, there is
154 no significant difference between *D. longispina* and *C. quadrangula*.

155

156 **4. Discussion**

157 Many studies revealed that resting egg of cladoceran species might be used for
158 species identification (Brendonck and De Meester, 2003; Vandekerkhove et al., 2004).
159 However, there are only two recently published literature describing resting eggs of
160 cladocera (Vandekerkhove et al., 2004 and Benzie, 2005) and information presented in
161 this literature is too short and not detailed. Also there are only one or two sentences
162 about resting eggs of *Daphnia magna* and *D. longispina* in the guide book by Benzie
163 (2005). This study describes resting eggs of three of about 800 cladoceran species
164 (Kotov et al, 2013) in detailed and reveals if there are significant morphological
165 differences between resting eggs of cladoceran species.

166 This study also shows that ephippia shape of the three different species is
167 different from each other. While ephippia of *C. quadrangula* is semicircular, ephippia

of *D. longispina* is narrowing at postero-ventral side. *D. magna* has an ephippia in shape of straight 'D' letter. Although *C. quadrangula* has one egg which is located parallel to dorsal margin of ephippium, other two species carry two eggs which is perpendicularly and horizontally to dorsal margin. In addition to, *D. magna* differs by anterior and posterior projections from other two species. Vandekerkhove et al. (2004) stated that morphology of ephippium is predominantly determined by the shape of active animals. Our findings also indicate that resting egg shapes differs according to species.

Stereo microscope analysis indicated that the background colours of ephippia of these three species also differ from each other. Resting egg colours of *C. quadrangula* (whitish cream background and orange brown egg) is completely different from other two species. Ephippia of *D. longispina* is covered with a transparent outer cover. While background colour of ephippia of *D. longispina* ranges from white to light brown, background colour of ephippia of *D. magna* is cream. Egg chambers of resting egg of *D. longispina* are also darker brown than *D. magna*.

As mentioned above, colours of ephippia and eggs enclosed in ephippia of these three species differ from each other. These differences may help to identify cladoceran species from resting eggs.

Benzie (2005) indicated that ephippia of *D. magna* is 1556 µm in length and 815 µm in width and ephippia of *D. longispina* is 794 µm in length and 550 µm in width. While ephippia size of *D. magna* is similar to our findings (1237± 119; mean±SD), ephippia size of *D. longispina* is bigger than our values (251±22; mean±SD) . This is probably because the measurements specified in the book belong to only one individual of each species. Jankowski and Straile (2003) emphasized that ephippium size decreases due to predation pressure. Boersma et al. (2000) also stated that "larger and older

192 females produce larger ephippia because ephippial eggs hatch in spring when the
193 abundance of size-selective predators is low".

194 Considering the results of our study, it is seen that different cladoceran species
195 produce resting eggs in different sizes. Mann-Whitney U test indicated that there is a
196 statistically significant difference between *D. magna* and *D. longispina*, *D. magna* and
197 *C. quadrangula* and *D. longispina* and *C. quadrangula* in terms of ephippia length and
198 width according to minimum $p<0.05$. Mann-Whitney U test also revealed statistically
199 significant difference between *D. magna* and *D. longispina*, *D. magna* and *C.*
200 *quadrangula* and *D. longispina* and *C. quadrangula* in terms of width and length of
201 eggs enclosed in ephippia according to minimum $p<0.05$. For that reason we suggest
202 that size differences might be used for identification of cladoceran species.

203 Because there was no study previously conducted relevant to size of eggs
204 enclosed in ephippia of these three species, data obtained from this study will shed light
205 on future works.

206 Consequently, in this study, it was revealed that ephippia and egg/eggs enclosed
207 in ephippia of *D. magna*, *D. longispina* and *C. quadrangula* has different characteristics
208 in terms of shape, colour and size. Some information about shape and size of ephippia
209 of these three species was reported in former taxonomical studies (Vandekerkhove et
210 al., 2004; Benzie, 2005). But, these information is short, and not detailed. This study
211 supplied detailed information about these features and statistic analysis also indicated
212 that sizes of these three species are statistically different from each other. In addition,
213 colour of ephippia and colour of eggs enclosed in ephippia of these three species were
214 described in detailed for the first time in this study and information about sizes of the
215 eggs enclosed in ephippia were revealed for the first time in this study. Results of this

study indicated that these features differ according to species and they can be used to distinguish these cladoceran species.

Acknowledges

We thank to Dr. Fatih Duman (Erciyes University, Biology Department) for statistic analysis.

REFERENCES

Benzie JAH (2005). The genus *Daphnia* (including Daphniopsis) (Anomopoda: Daphniidae), Kenobi Productions, Ghent, 2005. Backhuys Publishers, Leiden.

Boersma M, Boriss H, Mitchell SE (2000). Maternal effects after sexual reproduction in *Daphnia magna*. J Plankton Res 22: 279–285.

Brendonck L, De Meester L (2003). Egg banks in freshwater zooplankton: evolutionary and ecological archives in the sediment. Hydrobiologia 491: 65–84.

Caceres C.E., (1998). Interspecific Variation in the abundance, production, and emergence of *Daphnia* diapausing eggs. Ecology 79:1699-1710.

Conde-Porcuna JM, Valdés FJ, Romo S, Pérez-Martínez C (2011). Ephippial and subitaneous egg abortion: relevance for an obligate parthenogenetic *Daphnia* population. J Limnol 70: 69-75.

Davison J (1969). Activation of the ephippial egg of *Daphnia pulex*. J Gen Physiol 53: 562-575.

Haghparast S, Shabani A, Shabanpour B, Hoseini SA (2012). Hatching requirements of *Daphnia magna* Straus, 1820, and *Daphnia pulex* Linnaeus, 1758, diapausing eggs from Iranian populations in vitro. J Agric Sci Technol 14: 811-820.

240 Jankowski T, Straile D (2003). A comparison of egg-bank and long-term plankton
241 dynamics of two *Daphnia* species, *D. hyalina* and *D. galeata*: Potentials and limits
242 of reconstruction First publ. in: Limnol Oceanogr 48: 1948–1955.
243 Kotov A, Forró L, Korovchinsky NM, Petrusek A 2013 Jan 25, World checklist of
244 freshwater Cladocera species. World Wide Web electronic publication. Available
245 online at http://fada.biodiversity.be/group/show/17
246 Panarelli EA, Casanova SMC, Henry R (2008). The role of resting eggs in the recovery
247 of zooplankton community in a marginal lake of the Paranapanema River (São
248 Paulo, Brazil), after a long drought period. Acta Limnol Bras 20: 73-88.
249 Reid VA, Carvalho GR, George DG (2000). Molecular genetic analysis of *Daphnia* in
250 the English Lake District: species identity, hybridisation and resting egg banks.
251 Freshwater Biol 44: 247-253.
252 Rother A, Pitsch M, Hulsmann S (2010). The importance of hatching from resting eggs
253 for population dynamics and genetic composition of *Daphnia* in a deep reservoir.
254 Freshwater Biol 55: 2319–2331
255 Slusarczyk M (2001). Food threshold for diapause in *Daphnia* under the threat of fish
256 predation. Ecology 82: 1089–1096.
257 Slusarczyk M, Pietrzak B (2008). To sink or float: the fate of dormant offspring is
258 determined by maternal behaviour in *Daphnia*. Freshwater Biol 53: 569–576.
259 Vandekerkhove J, Declerck S, Vanhove M, Brendonck L, Jeppesen E, Conde-Porcuna
260 JM, De Meester L, (2004). Use of ephippial morphology to assess richness of
261 anomopods: potentials and pitfalls. J Limnol 63: 75-84.

Vanickova I, Seda J, Petrusek A (2010). The stabilizing effect of resting egg banks of the Daphnia longispina species complex for longitudinal taxon heterogeneity in long and narrow reservoirs. Hydrobiologia 643: 85–95.

Table 1. Resting eggs sizes of three species (*Daphnia magna*, *D. longispina*, *Ceriodaphnia quadrangula*)

Resting eggs		**Min**	**Max**	**Mean**	**SD**
Daphnia magna	**Length (µm)**	1013	1526	1237	119
	Width (µm)	479	817	625	74
Daphnia longispina	**Length (µm)**	310	539	405	45
	Width (µm)	260	410	330	29
Ceriodaphnia quadrangula	**Length (µm)**	267	381	317	23
	Width (µm)	184	276	222	17

279

280

281

282

283

284

285

286

287

288 **Table 2.** Size of eggs enclosed in ephippium of three species (*Daphnia magna*, *D.*

289 *longispina*, *Ceriodaphnia quadrangula*)

Eggs enclosed in ephippium		**Min**	**Max**	**Mean**	**SD**
Daphnia magna	**Length (µm)**	390	550	472	40
	Width (µm)	247	375	315	30
Daphnia longispina	**Length (µm)**	206	294	251	22
	Width (µm)	98	151	135	11
Ceriodaphnia quadrangula	**Length (µm)**	200	287	245	20
	Width (µm)	77	156	121	13

290

291

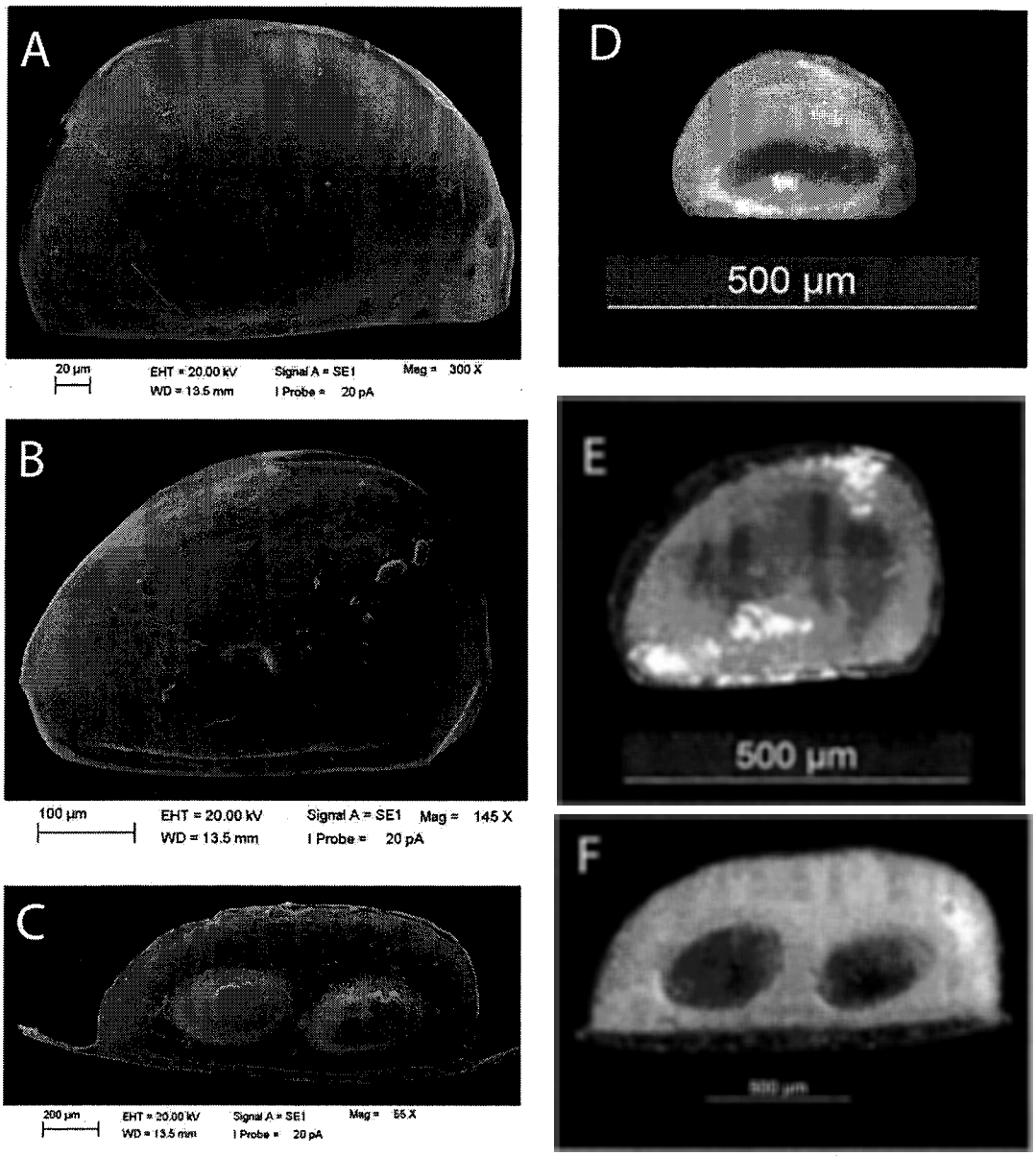
292

293

294

295

Figure 1: Resting egg photos of three species (*Daphnia magna*, *D. longispina*, *Ceriodaphnia quadrangula*) A: SEM photo of *C. quadrangula*, B: SEM photo of *D. longispina*, C: SEM photo of *D. magna*, D: Light microscope photo of *C. quadrangula*, E: Light microscope photo of *D. longispina*, F: Light microscope photo of *D. magna*



315

316 Figure 1

317

NEW DOCUMENT

Freshwater Biology (2004) **49**, 286–295

Salinity tolerance of diapausing eggs of freshwater zooplankton

SARAH A. BAILEY,* IAN C. DUGGAN,* COLIN D.A. VAN OVERDIJK,* THOMAS H. JOHENGEN,† DAVID F. REID‡ AND HUGH J. MACISAAC*

**Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, Canada*

†Cooperative Institute for Limnology and Ecosystems Research, University of Michigan, Ann Arbor, MI, U.S.A.

‡National Oceanic and Atmospheric Administration, Great Lakes Environmental Research Laboratory, Ann Arbor, MI, U.S.A.

SUMMARY

1. Many freshwater zooplankton produce diapausing eggs capable of withstanding periods of adverse environmental conditions, such as anoxia, drought and extreme temperature. These eggs may also allow oligostenohaline species to survive increased salinity during periods of tidal flux or evaporation, and here we test the ability of diapause eggs to withstand such conditions.

2. Salinity tolerance may also enable organisms to invade new environments. The increased rate of introduction of non-indigenous species to the Laurentian Great Lakes since 1989, when ballast water exchange regulations (to replace fresh/brackish water at sea with full seawater) were first implemented for transoceanic vessels, has stimulated studies that explore mechanisms of introduction, other than of active animals, in ballast water. One hypothesis proposes that freshwater organisms transported in ballast tanks as diapausing eggs may be partially responsible for the increased rate of species introduction, as these eggs may tolerate a wide array of adverse environmental conditions, including exposure to saline water.

3. We collected ballast sediments from transoceanic vessels entering the Great Lakes, isolated diapausing eggs of three species (*Bosmina liederi*, *Daphnia longiremis* and *Brachionus calyciflorus*), and measured the effect of salinity on hatching rate. In general, exposure to salinity significantly reduced the hatching rate of diapausing eggs. However, as non-indigenous species can establish from a small founding population, it is unclear whether salinity exposure will be effective as a management tool.

Keywords: ballast water exchange, biological invasion, hatching rates, resting eggs, salinity tolerance

Introduction

The introduction of non-indigenous species is a potent agent of biodiversity change, particularly for lake ecosystems (Sala *et al.*, 2000) and measures are urgently needed to identify and eliminate the vectors that transport them. Ballast water is recognised as the single most important vector for species introduction to aquatic habitats. Approximately 10 billion tonnes of ballast water (and its associated biota) are transferred annually between global ports, providing the primary means of transport and introduction of non-indigenous aquatic biota to ecosystems, including bacteria, dinoflagellates, phytoplankton, zooplankton and fish (Rigby, Hallegraeff & Sutton, 1999; Ruiz *et al.*, 2000). Transoceanic shipping accounts for 77% of the species introduced to the Laurentian Great Lakes since 1970 (Ricciardi, 2001). To reduce this threat, voluntary regulations were enacted in 1989, and mandated in 1993, that effectively require inbound vessels to exchange fresh or brackish ballast water with open-ocean saltwater if that water is to be discharged in the

Correspondence: Sarah A. Bailey, Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario, N9B 3P4, Canada. E-mail: sarahbailey@canada.com

Great Lakes (United States Coast Guard, 1993). Despite these regulations, the rate at which new species have been recorded in the Great Lakes tripled between 1989 and 1999 compared with the preceding 40 years (Grigorovich *et al.*, 2003). Increased sampling effort and time lags between establishment and discovery of non-indigenous species may partially account for this pattern (Costello & Solow, 2003; Grigorovich *et al.*, 2003), although modelling exercises indicated that ballast water exchange offers only incomplete protection and is least successful for species with benthic or dormant stages contained within ballast sediments (MacIsaac, Robbins & Lewis, 2002). Alternatively, the recent surge in non-indigenous species may be because of the presence of live or dormant organisms contained in the residual ballast of ships declaring 'no ballast on board', which are exempt from the regulations (MacIsaac *et al.*, 2002; Bailey *et al.*, 2003). These vessels carry tonnes of residual sediment and ballast water, and dominate trade inbound to the Great Lakes (Colautti *et al.*, 2003).

Many freshwater zooplankton, including copepods, cladocerans and rotifers, produce diapausing or 'resting' eggs during annual population cycles. These dormant stages probably evolved as an adaptation to periods of adverse environmental conditions, including anoxia, drought and extremely low or high temperature (Gilbert, 1974; Hairston, 1996; Hairston & Cáceres, 1996; Williams, 1998). These eggs could also provide temporal escape from unfavourable salinity, facilitating the intercontinental transfer of freshwater species in sediments of ballast tanks, even those subjected to ballast water exchange. Some of the species recently recorded in the Great Lakes are euryhaline endemics of the Ponto-Caspian region of southeast Europe, which may have been transported as resting stages in ballast water and/or sediments (Ricciardi & MacIsaac, 2000; Reid & Orlova, 2002). While the salinity tolerance of some juvenile and adult freshwater cladocerans and rotifers has been examined (e.g. Miracle & Serra, 1989; Teschner, 1995; Hall & Burns, 2002), very little is known of the salinity tolerance of the diapausing eggs of freshwater taxa. Consequently, it is difficult to infer whether invertebrates capable of producing diapausing eggs could circumvent the salinity 'filter' imposed on potential Great Lakes invaders by ballast water exchange.

In this study, we examine the effect of salinity on the hatching rate of diapausing eggs of the cladocerans *Bosmina liederi* De Melo & Hebert and *Daphnia longiremis* Sars, and the rotifer *Brachionus calyciflorus* Pallas, common inhabitants of ballast sediments of transoceanic vessels entering the Great Lakes. While these three species are native to the Great Lakes, their presence in residual ballast sediments suggests that they are representative of the types of organisms that pose a potential risk of invasion. Bailey *et al.* (2003) tested the viability of diapausing eggs recovered from ballast sediments and noted a tendency for reduced viability with high pore water salinity, although this relationship was not tested directly. Here, we test the hypothesis that diapausing eggs of freshwater zooplankton will be destroyed by exposure to saline water.

Methods

Sample collection

Residual sediments were collected from five transoceanic vessels entering the Great Lakes in 'no ballast on board' status in December 2000, May, August and December 2001, and in June 2002. Ships were sampled at the ports of Hamilton, Thorold and Toronto, Ontario, Canada, and Cleveland, OH, U.S.A. Residual sediment was collected from at least one ballast tank per ship, with additional tanks sampled depending upon availability and the ease and safety of access. Sediments were collected along longitudinal shell frames that trapped sediment in areas away from drainage flows. Approximately 4 kg of sediment (in total) were collected from at least five areas within each tank and placed in a single container. These composite samples were stored in the dark at 4 °C until experimentation. The salinity of residual sediment pore water, separated from sediment by centrifugation at approximately 3300 *g* (approximately 32 360 m s^{-2}) for 15 min, was measured using an optical refractometer (F. Dobbs, Old Dominion University, Norfolk, VA, U.S.A.).

Egg density counts

After thorough mixing, four 40-g subsamples (wet weight) were taken from each sample and preserved in 95% ethanol. Subsamples were each washed

through a 45 μm sieve to remove fine sediment. Diapausing eggs were subsequently separated from the coarse sediment using the colloidal silica Ludox® HS 40 (Burgess, 2001) and counted under a dissecting microscope.

Hatching experiments

Sediments were stored in plastic containers in the dark at 4 °C for at least 4 weeks to allow a refractory period before hatching experiments commenced (see Grice & Marcus, 1981; Schwartz & Hebert, 1987). After this time, diapausing eggs were removed from sediment using a sugar flotation method (Bailey *et al.*, 2003). Briefly, sediment was processed through a 45 μm sieve and washed into centrifuge tubes using a 1 : 1 (w : v) mixture of sucrose and water. After centrifugation (5 min at approximately ~27 *g*) the supernatant was decanted and rinsed thoroughly with water through 45 μm mesh before being transferred to a counting dish. Diapausing eggs were immediately recovered from the supernatant and sorted by size and gross morphology under a dissecting microscope, selecting only fully intact, apparently healthy eggs. A single, replicated experiment was conducted on the most abundant egg type (*Brachionus* or *Daphnia* species) for each of five tanks. For sediment from ship 1, in which *Brachionus budapestinensis* Daday eggs dominated (see Table 1), experiments were attempted using *B. budapestinensis*, but were abandoned owing to loss of eggs over time because of their extremely small size. Experiments were therefore conducted on a subdominant species, *B. liederi*. All other eggs were incubated at 0‰ (parts per thousand salinity) for identification purposes only. Occasionally, two or three species hatched during a single trial (see Table 1). These secondary species always contributed <1% of the total number of hatchlings. In total, 11 species hatched, although only three were used in the replicated experiments. Four trials were conducted with the rotifer *B. calyciflorus* and one trial each for the cladocerans *D. longiremis* and *B. liederi*.

Eggs used in the experiments were separated into 20 replicates of 20 eggs each, and placed into vials containing 15 mL of sterile medium (0, 8, 16, or 32‰) representing incremental efficiencies of ballast exchange. Five replicates were placed into each

Table 1 List of species hatched from ballast sediments through quantitative and qualitative hatching studies

Ship	No. of replicates	Species	No. eggs per 40 g	Pore water salinity (‰)
1 (FP)	N/A	*Brachionus budapestinensis*	92	2
	5	*Bosmina liederi*	56	
	N/A	*Brachionus calyciflorus*	52	
	N/A	*Daphnia longiremis*	6.3	
	N/A	*Daphnia ambigua* Scourfield*		
2 (DB)	5	*Daphnia longiremis*	391	10
	N/A	*Daphnia ambigua**		
3 (DB)	5	*Brachionus calyciflorus*	100	35
	N/A	*Brachionus quadridentatus* f. *rhenanus* (Lauterborn)*		
	N/A	*Brachionus urceolaris* Müller*		
	N/A	*Brachionus budapestinensis*	56.5	
	N/A	*Brachionus angularis* Gosse*		
4 (DB)	5	*Brachionus calyciflorus*	57.8	4
	N/A	*Daphnia magna* Straus	1.5	
	N/A	*Diaphanosoma* sp.	<1	
4 (FP)	5	*Brachionus calyciflorus*	119.5	20
5 (DB)	5	*Brachionus calyciflorus*	187.8	10
	N/A	*Asplanchna brightwelli* Gosse	1.5	

Species with N/A replicates were not used during experimentation, and were hatched only for identification purposes. Ship tanks are identified by type: FP, forepeak tank; DB, double-bottom tank.

*Denotes secondary species hatched from single morphological egg type listed immediately above.

salinity treatment at 20 °C (photoperiod 16 h light : 8 h dark), resulting in an experimental design using 400 eggs per trial. The 0‰ treatment was considered a control to assess maximum viability for these freshwater species. Synthetic pond water (Hebert & Crease, 1980) or diluted, filtered, natural seawater (collected from a vessel transiting the Great Lakes loaded with ocean water ballast, filtered through 0.2 μm Whatman number 5 paper filter) were used as hatching media. Vials were checked for emergence every 24 h for 10 days, with all hatched individuals removed daily. Media were refreshed on day 5. On day 10 all remaining eggs were transferred to synthetic freshwater media by pipette to examine hatching rates after salinity exposure. Again, the number of hatchlings was recorded daily. Negative controls containing only treatment media were kept in each treatment group to detect any introductions of organisms from the environment. We chose the 10 day hatching period after exposure for two reasons. First, previous experiments indicated that 96% of hatching occurs within the first 10 days of trials run for 20 or 30 days in the manner described above (Bailey *et al.*, 2003; unpublished data). Secondly, the typical transit time of a 'no ballast on board' vessel carrying Great Lakes water within the lake system is 7–10 days. If the uptake of Great Lakes water does induce diapausing eggs contained in ballast sediments to hatch (as suggested by Bailey *et al.*, 2003), this is the period of greatest risk.

Variation in the cumulative proportion of diapausing eggs hatched between treatments was analysed using a one-way ANOVA with repeated measures using Systat 8.0 (SPSS, 1998; SPSS, Chicago, IL, U.S.A.). Tukey's multiple comparison test was performed on the total proportion of eggs hatched to determine the impact of salinity on hatching rate. As emergence was inhibited at higher salinities, analyses were conducted on the 10-day hatching segment for each treatment, depending on the timing of emergence (i.e. days 0–10 for 0 and 8‰ treatments were compared with days 10–20 for 16 and 32‰; if no hatching occurred during days 0–10 for the 8‰ treatment, then days 10–20 were used). Only days when hatching occurred in at least one of the replicates were analysed. The proportion of eggs hatched was normalised using an arcsine square root transformation before analysis.

Results

Hatching experiments were conducted on zooplankton diapausing eggs isolated from residual ballast sediment collected from six tanks on five vessels. Salinity of sediment pore water varied from 2 to 35‰ (Table 1). Diapausing egg densities of dominant taxa were high (>50 eggs per 40 g sediment, Table 1). Eggs were induced to hatch in all experiments, with hatching rates ranging from 16 to 89% in the 0‰ treatment (Fig. 1). In each experiment the proportion hatching declined with increasing salinity. No organisms were recorded in the negative control vials. *Brachionus calyciflorus* generally began to hatch within 24 h of incubation at 0‰, while for the cladocerans *B. liederi* and *D. longiremis* emergence began at day 3 (Fig. 1). Development also began promptly in the 8‰ treatments, with *B. calyciflorus* hatching in three out of four trials by day 5. In addition, development of eye-stage embryos was recorded by day 5 in the 8‰ treatments for 50 and 90% of *Daphnia* and *Bosmina* eggs, respectively (see Fig. 2a). Apparently, these species could not tolerate emergence into brackish water, as development always stopped before emergence from eggs was complete. None of the eye-stage embryos recorded in 8‰ treatment continued development after the medium was replaced with 0‰ water on day 10. Conversely, no organisms hatched or completed significant development during the 10 days of exposure at either of the two higher salinities (i.e. 16 and 32‰, Fig. 2b,c), although some lipid accumulation was noted in the 16‰ treatment. Rather, emergence occurred in these treatments only after brackish or saltwater media were exchanged for 0‰ water (Fig. 1). After exchange, hatching rates among experiments varied between 0–31 and 0–78% for the 16 and 32‰ treatments, respectively. *Bosmina liederi* was the only species tested for which no hatching occurred after exposure to any of the salinity (>0‰) treatments.

The difference in hatching rate between treatments was highly significant for all trials ($P < 0.05$, ANOVA, Table 2). All trials exhibited divergence of hatching rates over time, as time × treatment interaction terms were significant ($P < 0.0001$, ANOVA, Table 2). The proportion of eggs hatched was higher in the 0‰ treatment for eggs recovered from ships 1–4 (Fig. 1a-e; $P < 0.05$, Tukey *post hoc* test). The hatching rates of *B. calyciflorus* for the 0 and 32‰ treatments for ship 5

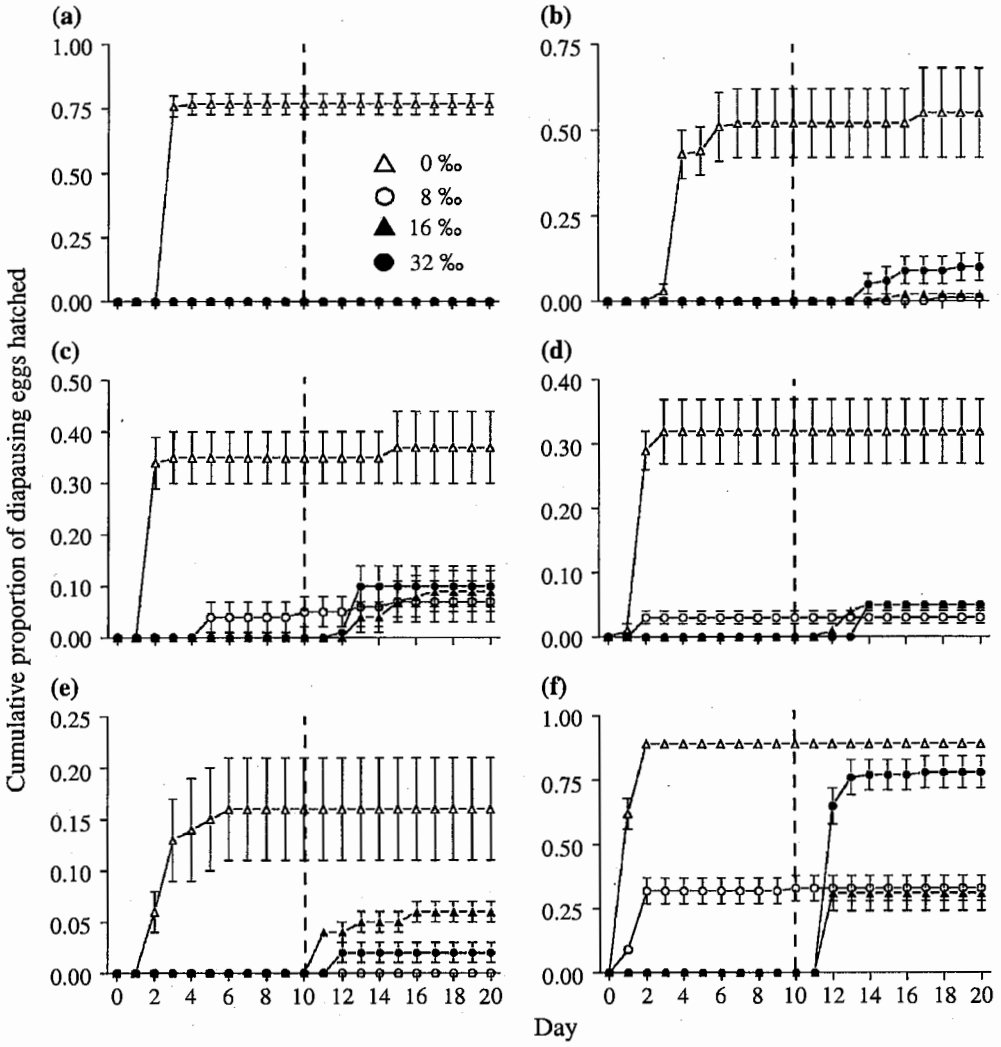


Fig. 1 Mean (±SE) cumulative proportion of diapausing eggs hatched under salinity treatments, by species. (a) *Bosmina liederi* (ship 1), (b) *Daphnia longiremis* (ship 2), (c) *Brachionus calyciflorus* (ship 3), (d) *B. calyciflorus* (ship 4-DB), (e) *B. calyciflorus* (ship 4-FP) and (f) *B. calyciflorus* (ship 5). After 10 days (dotted vertical line) all unhatched eggs in each treatment group were transferred to 0‰ media. Note scale difference for each ordinate. Error bars <0.03 are hidden by graph symbol.

were significantly higher than for the other two treatments (Fig. 1f; $P < 0.001$, Tukey *post hoc* test).

Discussion

To date, investigations of the salinity tolerance of freshwater zooplankton have been limited to measuring direct effects on growth and survival (e.g. Miracle & Serra, 1989; Teschner, 1995; Hall & Burns, 2002), or examining species richness and composition in waterbodies of varying salinity (Frey, 1993; Brain, Fourie & Shiel, 1995). These approaches have not considered diapausing egg stages, probably resulting in an underestimate of the range of salinities a particular taxon can tolerate, particularly in instances where salinity varies temporally. In this study, we have demonstrated that the hatching rate of diapausing eggs is reduced by exposure to saline conditions. The ability of diapausing eggs to tolerate fluctuations in salinity may stem from an evolutionary history in temporary habitats, which generally fluctuate more in their physical and chemical environment than adjacent, permanent ones (Williams, 1998).

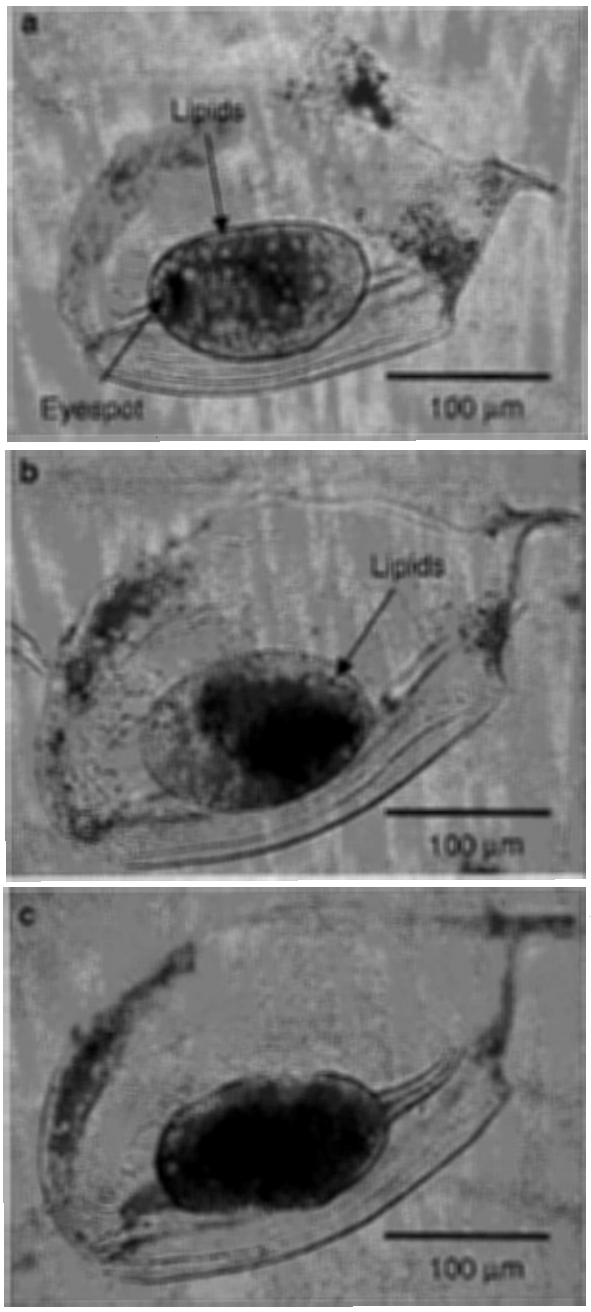


Fig. 2 Condition of diapausing eggs of *Bosmina liederi* after 10 days at each treatment. (a) 8‰, aborted eyed-embryo, (b) 16‰, little differentiation and (c) 32‰, no change. Scale bars (100 µm) are included on each image.

Of the species examined here, diapausing eggs of *B. liederi* appear to exhibit the lowest salinity tolerance with no hatching after exposure to saltwater. *Daphnia longiremis* exhibited a modest degree of salinity tolerance, as a small proportion of diapausing eggs hatched following saltwater exposure. *Brachionus calyciflorus* demonstrated the widest tolerance, with up to 78% eggs hatching after saltwater exposure. Interestingly, *B. calyciflorus* also displayed a wider salinity tolerance as adults than either *B. liederi* or *D. longiremis*. Although typically considered to 'prefer' freshwater, *B. calyciflorus* is frequently observed in brackish waters (e.g. Brain *et al.*, 1995; Park & Marshall, 2000). It is also the only species in these trials that successfully hatched into 8‰ salinity during days 0–10, albeit at rates significantly lower than in freshwater. This finding is consistent with observations of Snell *et al.* (1991), who reported an approximately 40% reduction in the hatching rate of *B. calyciflorus* at 8‰ as compared with 2‰ growth media.

The 'maximum' hatching rates observed at 0‰ ranged from 16–89%. Bailey *et al.* (2003) suggested that pore water salinity might be negatively correlated with hatching success. Our study also found a low hatching rate for eggs recovered from sediments with a pore water salinity of 20‰ or higher (16 and 37%). However, lower pore water salinity (≤10‰) did not guarantee a high hatching rate (i.e. 32% hatch for 4‰), so other factors (such as age of eggs, duration of diapause or hatching cues) are probably involved. In addition, the effects seen in both studies may be impacted by the wide salinity tolerance of *B. calyciflorus* (i.e. 10‰ is only slightly above the natural range for this species, resulting in a high hatching rate at intermediate salinity). Alternatively, pore water salinity measured at the time of collection may not be a good indicator of egg history; eggs may have been retained in ballast sediments for years, experiencing widely varying salinity, of which only the most recent may be reflected by pore water salinity. Nevertheless, while it is possible that the 'maximum' hatching rate (and subsequent reductions in hatching rate) measured in this study may be affected by previous exposures, our results do indicate the effectiveness of ballast water exchange because the sediments carried in transoceanic vessels originate from ports of varying salinity.

Hatching during days 10–20, following transfer to 0‰ medium, occurred mainly in the 16 and 32‰ treatments. Very little hatching occurred during this period in either the 0 or 8‰ treatments, with individuals emerging only from eggs that had not visibly developed during the first 10 days. This suggests that

Table 2 Analysis of variance with repeated measures demonstrating the effect of salinity treatment on the hatching rate of diapausing eggs

Ship	Organism	ANOVA effects [*F* value (d.f.)]		
		Treatment	Time	Time × treatment
1 (FP)	*Bosmina liederi*	32.56 (3,16)***	43.48 (3,48)***	16.98 (9,48)***
2 (DB)	*Daphnia longiremis*	368.97 (3,16)***	353.05 (9,144)***	350.90 (27,144)***
3 (DB)	*Brachionus calyciflorus*	19.78 (3,17)***	41.08 (7,119)***	10.39 (21,119)***
4 (DB)	*Brachionus calyciflorus*	36.60 (3,16)***	47.26 (4,64)***	15.10 (12,64)***
4 (FP)	*Brachionus calyciflorus*	6.15 (3,16)*	14.97 (6,96)***	6.54 (18,96)***
5 (DB)	*Brachionus calyciflorus*	35.25 (3,16)***	322.04 (4,64)***	22.47 (12,64)***

Data were arcsine square root transformed prior to analysis. Significance levels for *F*-values: *$P < 0.05$; ***$P < 0.0001$. Ship tanks are identified by type: FP, forepeak tank; DB, double-bottom tank.

the *Bosmina* and *Daphnia* eye-stage embryos that developed by day 5 at 8‰ were no longer viable. It is possible that a salinity of 8‰ is sufficiently low for the initiation of egg development in freshwater species, but too high for complete development and emergence to occur. In contrast, no development in these genera was apparent in eggs exposed to 32‰ water, thus there remained a 'bank' of viable embryos left to emerge following transfer to freshwater media. Although both *B. liederi* and *D. longiremis* displayed this trend, we cannot explain why only *Daphnia* eggs hatched after exposure to 32‰. However, this trend was also observed for *B. calyciflorus*, with a higher emergence rate after exposure to higher rather than to lower salinity during the latter half of the experiment, particularly for eggs from ship 5. A similar phenomenon was observed by Lutz, Marcus & Chanton (1994), who exposed copepod resting eggs to variable oxygen conditions. They noted that low oxygen concentrations were more detrimental to egg viability than total anoxia because metabolism was completely shut down during anoxia but not under low oxygen conditions. Thus, there appears to be greater interaction between the embryo and the environment under nearly favourable conditions than under extreme conditions. However, it is also possible that the transfer of eggs from 32 to 0‰ acted as a stronger hatching cue than the transfer of eggs from 8 or 16 to 0‰. If this is the case, then subjecting diapause eggs to ballast water of 32‰ may actually promote mass hatching once the eggs are returned to freshwater conditions.

Charmantier & Charmantier-Daures (2001) suggested that rehydrated *Artemia* embryos are protected from high salinity by the cyst envelope that is permeable to water but impermeable to ions. However, salinity and temperature are known to interact in their effects on tolerance, with temperature affecting metabolic rate, ion uptake rate, and membrane permeability (Lee & Bell, 1999). Our experiments explored salinity tolerance at 20 °C, arguably a more challenging environment than exposure at a lower temperature for temperate species. It will be necessary to conduct future trials at a variety of temperatures to deduce the interaction between temperature and salinity on diapausing egg viability.

The variation in hatching rate seen among *B. calyciflorus* trials after exposure (day 10–20) may have resulted from the disparate histories of the populations tested, as indicated by pore water salinity of ballast sediments. Of particular interest was the hatching rate of *B. calyciflorus* collected from ship 5, as 78% eggs hatched successfully after exposure to salinities up to open-ocean levels (i.e. 32‰). In contrast, hatching rates of the other three *B. calyciflorus* populations were significantly reduced after similar exposure (<10%). It is possible that salinity experienced during diapause egg formation may influence the range of salinities eggs can survive while dormant, much like it affects the optimal salinity for the initiation of hatching for the euryhaline rotifer *Brachionus plicatilis* Müller (Gilbert, 1974). We were unable to explore this hypothesis, as the origins of the diapausing eggs in this study are unknown. Future studies using clonal populations from both permanent and temporary habitats may help clarify this possibility.

In general, <10% of *Daphnia* and *Brachionus* eggs hatched after salinity exposure in our experiments. Nevertheless, considering the high egg density in ballast sediments (10^4–10^5 eggs m^{-2} using 1.6 g cm^{-3}

conversion factor for wet sediment), large populations of viable zooplankton eggs may remain after salinity exposure. While it is possible that a longer exposure may have reduced egg viability further, the length of transoceanic crossings will generally not permit longer exposure regimes. As only a small 'seed population' is necessary to establish a cohort of reproductive individuals, and given that the maximum density of diapausing eggs in natural populations ranges between 10^3 and 10^6 eggs m^{-2} (Hairston, 1996), new populations could establish when salinity returns to favourable values (i.e. when the vessel subsequently loads freshwater, or if the eggs get flushed into a freshwater environment). Hall & Burns (2001) suggest that resting eggs of *Boeckella hamata* Brehm, a freshwater copepod, are responsible for the recolonisation of the tidally-influenced Lake Waihola, New Zealand, after seasonal salinisation up to 4.8‰. The average hatching rate for resting eggs of *B. hamata* was only 2.3% under optimal conditions in the laboratory. Therefore, while ballast water exchange may reduce the viability of diapausing eggs by as much as 90% for some taxa, it apparently does not offer complete protection against non-indigenous species entering the Great Lakes by this mechanism. Interestingly, our study indicates that ballast water exchange using brackish water (e.g. 8‰) may have a larger impact on diapausing egg viability than 32‰; however, this effect would have to be weighed against the possibility of introducing live euryhaline species in water of lower salinity, for which ballast water exchange of 32‰ is decidedly more effective (Locke *et al.*, 1993; MacIsaac *et al.*, 2002).

Furthermore, most transoceanic vessels currently trading on the Great Lakes declare 'no ballast on board' status (Colautti *et al.*, 2003), and thus are exempt from ballast water exchange regulations (United States Coast Guard, 1993). MacIsaac *et al.* (2002) suggested that these vessels, collectively, may pose a higher invasion risk than vessels entering the system with saline ballast water owing to the abundance of viable diapausing eggs contained within residual sediments. Our results suggest that the risk posed by diapausing eggs present in sediments of these vessels could be reduced, but not eliminated, by introducing a lens of saltwater into the 'empty' ballast tanks similar to ballast exchange.

Sala *et al.* (2000) suggested that lakes will experience very steep declines in biodiversity this century owing to biotic exchange, land use change and climate change. The salinity of endorheic freshwater habitats is likely to increase during summer months as water inputs decline and evaporation increases (Schindler, 1997, 2001). In addition, coastal lakes and freshwater habitats upstream from tidal estuaries may suffer periodic salinisation as pulsing surges of saltwater seep inland owing to evaporation and anthropogenic diversion of freshwater (Jones, 1994; Hall & Burns, 2003). The persistence of populations through salinity fluctuations by means of diapausing eggs could have profound implications on the extent of biodiversity loss during habitat change. Species incapable of tolerating changing salinity could be replaced by taxa tolerant of brackish or saline conditions (Schindler, 1997); however, this study demonstrates that some populations may be capable of tolerating enhanced fluctuations in habitat salinity, providing a mechanism for enriching biodiversity if the habitat returns to freshwater conditions.

Acknowledgments

We are grateful to F. Dobbs for providing pore water salinity data, and D. Gray for laboratory assistance. The Shipping Federation of Canada, the Great Lakes Shipping Association, and their ship agents, masters, officers and crew were all very accommodating to our needs. This project was supported by an NSERC Industrial Postgraduate Scholarship, in partnership with the Shipping Federation of Canada, an Ontario Graduate Scholarship and an Ontario Federation of Anglers and Hunters Conservation Grant to SAB, and by a Premier's Research Excellence Award to HJM. This work was conducted under the multi-institutional Great Lakes NOBOB Project funded by the Great Lakes Protection Fund, the National Oceanic and Atmospheric Administration (NOAA), the U.S. Environmental Protection Agency, and the U.S. Coast Guard. The project was co-managed by the Cooperative Institute for Limnology and Ecosystems Research and the NOAA Great Lakes Environmental Research Laboratory and sponsored under cooperative agreement NA17RJ1225 from the Office of Oceanic and Atmospheric Research, NOAA (GLERL Contribution 1293).

References

Bailey S.A., Duggan I.C., van Overdijk C.D.A., Jenkins P.T. & MacIsaac H.J. (2003) Viability of invertebrate diapausing eggs collected from residual ballast sediment. *Limnology and Oceanography*, **48**, 1701–1710.

Brain C.K., Fourie I. & Shiel R.J. (1995) Rotifers of the Kalahari Gemsbok National Park, South Africa. *Hydrobiologia*, **313/314**, 319–324.

Burgess R. (2001) An improved protocol for separating meiofauna from sediments using colloidal silica sols. *Marine Ecology Progress Series*, **214**, 161–165.

Charmantier G. & Charmantier-Daures M. (2001) Ontogeny of osmoregulation in Crustaceans: The embryonic phase. *American Zoologist*, **41**, 1078–1089.

Colautti R.I., Niimi A.J., van Overdijk C.D.A., Mills E.L., Holeck K. & MacIsaac H.J. (2003) Spatial and temporal analysis of transoceanic shipping vectors to the Great Lakes. In: *Invasion Pathways: Analysis of Invasion Patterns and Pathway Management*. (Eds G.M. Ruiz, J.T. Carlton & R.N. Mack), pp. 227–246. Island Press, Washington, D.C.

Costello C.J. & Solow A.R. (2003) On the pattern of discovery of introduced species. *Proceedings of the National Academy of Sciences USA*, **100**, 3321–3323.

Frey D.G. (1993) The penetration of cladocerans into saline waters. *Hydrobiologia*, **267**, 233–248.

Gilbert J.J. (1974) Dormancy in rotifers. *Transactions of the American Microscopical Society*, **93**, 490–513.

Grice G.D. & Marcus N.H. (1981) Dormant eggs of marine copepods. *Oceanography and Marine Biology Annual Review*, **19**, 125–140.

Grigorovich I.A., Colautti R.I., Mills E.L., Holeck K. & MacIsaac H.J. (2003) Ballast-mediated animal introductions in the Laurentian Great Lakes: retrospective and prospective analyses. *Canadian Journal of Fisheries and Aquatic Sciences*, **60**, 740–756.

Hairston N.G. Jr (1996) Zooplankton egg banks as biotic reservoirs in changing environments. *Limnology and Oceanography*, **41**, 1087–1092.

Hairston N.G. Jr & Cáceres C.E. (1996) Distribution of crustacean diapause: Micro- and macroevolutionary pattern and process. *Hydrobiologia*, **320**, 27–44.

Hall C.J. & Burns C.W. (2001) Hatching of *Boeckella hamata* (Copepoda: Calanoida) resting eggs from sediments of a tidally influenced lake. *New Zealand Journal of Marine and Freshwater Research*, **35**, 235–238.

Hall C.J. & Burns C.W. (2002) Mortality and growth responses of *Daphnia carinata* to increases in temperature and salinity. *Freshwater Biology*, **47**, 451–458.

Hall C.J. & Burns C.W. (2003) Responses of crustacean zooplankton to seasonal and tidal salinity changes in the coastal Lake Waihola, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, **37**, 31–43.

Hebert P.D.N. & Crease T.J. (1980) Clonal existence in *Daphnia pulex* (Leydig): another planktonic paradox. *Science*, **207**, 1363–1365.

Jones G. (1994) Global warming, sea level change and impact on estuaries. *Marine Pollution Bulletin*, **28**, 7–14.

Lee C.E. & Bell M.A. (1999) Causes and consequences of recent freshwater invasions by saltwater animals. *Trends in Ecology and Evolution*, **14**, 284–288.

Locke A., Reid D.M., van Leeuwen H.C., Sprules W.G. & Carlton J.T. (1993) Ballast water exchange as a means of controlling dispersal of freshwater organisms by ships. *Canadian Journal of Fisheries and Aquatic Sciences*, **50**, 2086–2093.

Lutz R.V., Marcus N.H. & Chanton J.P. (1994) Hatching and viability of copepod eggs at two stages of embryological development: anoxic/hypoxic effect. *Marine Biology*, **119**, 199–204.

MacIsaac H.J., Robbins T.C. & Lewis M.A. (2002) Modeling ships' ballast water as invasion threats to the Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, **59**, 1245–1256.

Miracle M.R. & Serra M. (1989) Salinity and temperature influence in rotifer life history characteristics. *Hydrobiologia*, **186/187**, 81–102.

Park G.S. & Marshall H.G. (2000) The trophic contributions of rotifers in tidal freshwater and estuarine habitats. *Estuarine, Coastal and Shelf Science*, **51**, 729–742.

Reid D.F. & Orlova M.I. (2002) Geological and evolutionary underpinnings for the success of Ponto-Caspian species invasions in the Baltic Sea and North American Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, **59**, 1144–1158.

Ricciardi A. (2001) Facilitative interactions among aquatic invaders: is an 'invasional meltdown' occurring in the Great Lakes? *Canadian Journal of Fisheries and Aquatic Sciences*, **58**, 2513–2525.

Ricciardi A. & MacIsaac H.J. (2000) Recent mass invasion of the North American Great Lakes by Ponto-Caspian species. *Trends in Ecology and Evolution*, **15**, 62–65.

Rigby G.R., Hallegraeff G.M. & Sutton C. (1999) Novel ballast water heating technique offers cost-effective treatment to reduce the risk of global transport of harmful marine organisms. *Marine Ecology Progress Series*, **191**, 289–293.

Ruiz G.M., Rawlings T.K., Dobbs F.C., Drake L.A., Mullady T., Huq A. & Colwell R.R. (2000) Global spread of microorganisms by ships. *Nature*, **408**, 49–50.

Sala O.E., Chapin F.S. III, Armesto J.J. *et al.* (2000) Global biodiversity scenarios for the year 2100. *Science*, **287**, 1770–1774.

Schindler D.W. (1997) Widespread effects of climatic warming on freshwater ecosystems in North America. *Hydrological Processes*, **11**, 1043–1067.

Schindler D.W. (2001) The cumulative effects of climate warming and other human stresses on Canadian freshwaters in the new millennium. *Canadian Journal of Fisheries and Aquatic Sciences*, **58**, 18–29.

Schwartz S.S. & Hebert P.D.N. (1987) Methods for the activation of the resting eggs of *Daphnia*. *Freshwater Biology*, **17**, 373–379.

Snell T.W., Moffat B.D., Janssen C. & Persoone G. (1991) Acute toxicity tests using rotifers. IV. Effects of cyst age, temperature and salinity on the sensitivity of *Brachionus calyciflorus*. *Ecotoxicology and Environmental Safety*, **21**, 308–317.

Teschner M. (1995) Effects of salinity on the life history and fitness of *Daphnia magna*: variability within and between populations. *Hydrobiologia*, **307**, 33–41.

United States Coast Guard. (1993) Ballast water management for vessels entering the Great Lakes. Code of Federal Regulations 33-CFR Part 151.1510.

Williams D.D. (1998) The role of dormancy in the evolution and structure of temporary water invertebrate communities. *Archiv für Hydrobiologie, Special Issues in Advanced Limnology*, **52**, 109–124.

(Manuscript accepted 28 December 2003)