Comparative Sensitivity of *Centroptilum triangulifer*, *Ceriodaphnia dubia* and *Daphnia magna* to standard Salt and Copper Toxicants

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EPA United States Environmental Protection Agency

Background

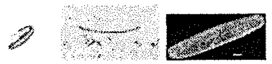
- In Mid Atlantic U.S. - Mayflies have been found to be sensitive to mine waste high in total dissolved solids (TDS)
- Organism chosen for this study was *Centroptilum triangulifer* an obligate parthenogenetic mayfly that inhabits slow flow or depositional areas in streams throughout the northeastern United States and eastern Canada (Sweeney and Vannote, 1984).
- Their life cycle is ~30 days at 25°C from newly hatched larvae to emergent adult. They lay 1000+ eggs per female and larvae normally feed on periphytic algae and diatoms.
- Previous studies have utilized mayflies in toxicity studies, and results indicate the organism may be very sensitive to certain toxicants. (e.g., Xie, Funk, Buchwalter 2009, Conley, Funk, Buchwalter 2009, Hassell, Kefford, Nugegoda 2006)
- Testing to date has used glass plates that have been colonized by diatoms and algae with local streams serving as a seed and water source.

Objectives

- Develop standard laboratory culture method for *C. triangulifer*
- Develop a standard toxicity test protocol for acute and chronic testing for *C. triangulifer*.
- Compare the sensitivity of *C. triangulifer* to standard testing organisms, *Ceriodaphnia dubia*, and *Daphnia magna*
- Deploy *C. triangulifer* in stream mesocosms to test sensitivity to simulated resource extraction TDS concentrations.

Methods

C. triangulifer Culture Methods
Diatom Stock Food Cultures



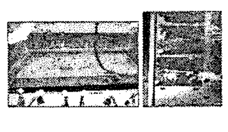
Mayamaea *Nitzschia* *Achnanthidium*

The following species of diatoms were chosen based on size range and abundance in previous culture studies using native stream diatoms:
Mayamaea atomus var. permitis (Hustedt),
Nitzschia cf. pusilla (Grunow), and
Achnanthidium minutissimum (Kützing).

Diatom Stock Cultures

- Autoclaved 2-L flask for each diatom species stock containing 1 L de-ionized water (Filtered Lab line can also be used here as a substitute)
- Add 20 ml algal starter culture and 0.4 ml each of Kent* – Proculture Professional F/2 Algal culture formula A and B then add 30mg of Sodium Metasilicate.
- Place on insulated stir-plate, stir moderately and allow growth for 10 days.
- Diatoms are checked for a minimum of 75% viability

Mixed Freshwater Diatom Slides:



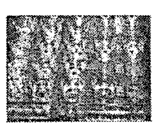
- 75 glass microscope slides are scratched with coarse grade sandpaper or purchased pre-scratched
- 15-L autoclavable container with 3 L of filtered de-chlorinated tap water supplemented with calcium chloride to obtain a hardness of 160 - 180 mg/L as $CaCO_3$
- 0.5 ml of Kent* – Proculture Professional F/2 Algal culture formula A and B Add 90mg of Sodium Metasilicate
- 45 ml of mixed freshwater diatoms (equal volumes of each species' stock culture) to each 15-L container.
- Allow diatoms to settle and grow on the glass slides for at least 10 days under 400 ft candles of light with a 12-hour day length.

C. triangulifer Nymph Rearing Method

- 15-L culture container with 4 L of labline water
- 60 newly hatched *C. triangulifer* (<24 hrs old) each in 2 containers (total=120)
- 8 slides of mixed freshwater diatom culture placed prior to the addition of *C. triangulifer* to avoid injury
- slides are replaced weekly, more often if needed
- change water 2x per week when the nymphs become visible to the eye
- 16 hours day light
- 25 °C temperature
- 50% relative humidity

Toxicity Tests

- KCl, NaCl and CuSO4 were chosen as standard reference toxicants based on EPA's Acute and Chronic Whole Effluent Toxicity Manual.
- Acute tests were run simultaneously with all three species. *C. triangulifer*, *Ceriodaphnia dubia*, and *Daphnia magna*
- Moderately Hard Reconstituted Water (EPA Acute Manual 2002) was used as control and dilution water
- Conductivity, pH, dissolved oxygen, and temperature were recorded daily for concentrations and renewal solution.



Conditions for Acute Testing All 3 Species

Acute Test Conditions	
Age of Organisms	< 24 hrs
Test Duration	48 hours
Test Temp	25° C +/- 1°C
Light Cycle	16 light/ 8 dark
Feeding	[illegible]
Water Renewal	Daily
Endpoint	Mortality
Test Criteria	<90% survival in control

Chronic Test Conditions

Duration of test, food, volume of solution and endpoint varied with each species as listed below

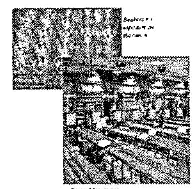
C. triangulifer
- 14 days duration
- Food: 0.2 ml daily 10 days, 0.4 ml daily for remaining 4 days
- Volume: 15ml
- Temperature: 25°C
- Endpoint: Growth as measure by head capsule width, length, and weight
- Test Criteria: >90% survival in controls

C. dubia
- 7 days duration
- Food: 0.2 ml Selenastrum and 0.1 ml FFAY daily
- Volume: 15ml
- Temperature: 25°C
- Endpoint: # of young reproduced
- Test Criteria: Avg. 15 young/female in 3 broods >80% survival in control

P. promelas
- 4 days duration
- Food: 0.3 ml Selenastrum and 0.2 ml alfalfa daily
- Volume: 50ml
- Temperature: 25°C
- Endpoint: Growth as weight
- Test Criteria: Mean dry weight of control 10x > dry weight for initials, >90% survival in the control

TDS MESOCOSM Study

- Run single organism tests in WET framework
- Simulate ecoStructure-Function to understand the role and response of small streams to changing nature of Point Sources.
- Study abiotic/biotic interactions as determinants of the role of small stream ecosystems in sustainable water management



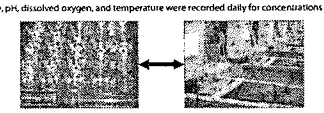
The Realized Excess TDS – that measured in the mesocosms

	PreDosing		Dosing							PostDose	
Date:	19-Jul	26-Jul	28-Jul	2-Aug	9-Aug	23-Aug	6-Sep	Dosing Period	Target	20-Sep	4-Oct
Sample ID	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	Average		mg/L	mg/L
E01	226	137	182	304	266	247	236	247	240	266	
E04	178	198	262	440	413	392	343	370	361	207	
E05	263	205	420	652	NA	586	703	590	501	242	
E06	268	224	631	796	732	774	778	742	695	302	
E03	254	218	1025	1363	1337	1322	1394	1288	1062	262	
E07	277	367	1883	2273	2181	2389	1602	2066	1812	222	
E02	272	195	3100	3810	3354	3595	4214	3615	3173	450	
E08	295	65	5714	7392	7252	7130	7337	6963	5655	229	
QC_200	284		300			286	235			173	
QC_6000	5767		5753			5861	5906			5893	
QC_Blank	16		75			53	2			2	

Excess TDS was: 63% Chloride, 24% Sodium; 9% Calcium

Mesocosm TDS Toxicity Tests

- Based on EPA's Acute and Chronic Whole Effluent Toxicity Manual
- Acute tests were run simultaneously with three species. *C. triangulifer*, *Ceriodaphnia dubia*, and *Daphnia magna*
- Moderately Hard Reconstituted Water (EPA Acute Manual 2002) was used as control and dilution water
- Chronic Tests were run on the bench top and ex-situ; 7, 14, or 20 day
- Conductivity, pH, dissolved oxygen, and temperature were recorded daily for concentrations and renewal solution

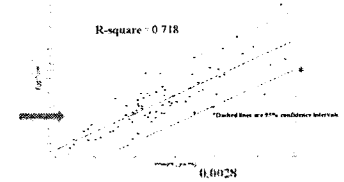


Results

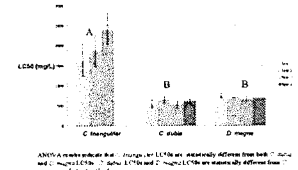
Culture Results Generational Survival and Mortality for *C. triangulifer*

F3	[illegible]	1	3.91%	[illegible]	1.46%	147	[illegible]
F4	[illegible]	[illegible]	[illegible]	0	0.00%	49	[illegible]
F5	[illegible]	1	1.31%	0	[illegible]	151	[illegible]
F6	[illegible]	8	3.37%	0	0.00%	153	[illegible]
F7	[illegible]	[illegible]	[illegible]	2	1.1%	40	[illegible]
F8	[illegible]	0	0.00%	1	[illegible]	182	[illegible]
F9	[illegible]	0	0.00%	16	[illegible]	95	[illegible]

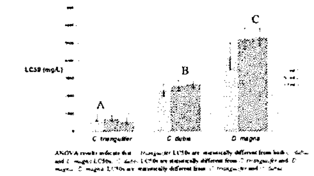
Regression Relationship Between pre Egg Laying Weight and Fecundity



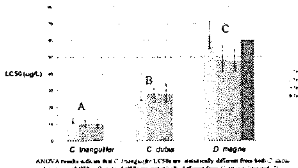
KCL Acute Results



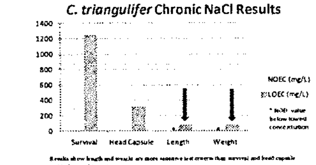
NaCl Acute Results



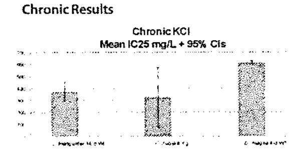
CuS04 Acute Results

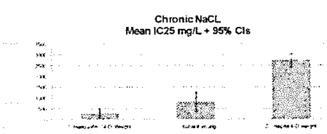


NaCl Chronic Results



Chronic Results





Acute Tests Results on Early Test TDS Samples in Distilled Water (DI) and East Fork River Water (EFR)

NaCl in DI

Conc.	TDS mg/L
MHRW	nd
#1	nd
#2	242
#3	383
#4	571
#5	946
#6	1696
#7	3009
#8	5540

	C. triangulifer		*C. dubia*		*D. magna*
LC50	[illegible]	LC50	3418.64	LC50	NA
95% UC	3173.55	95% UC	3859.5	NOEC	NA
95% LC	2331.68	95% LC	3028.14		
NOEC	1696	NOEC	1696		

NaCl in EFR

Conc.	TDS mg/L
MHRW	nd
#1	nd
#2	242
#3	383
#4	571
#5	946
#6	1696
#7	3009
#8	5540

	C. triangulifer		*C. dubia*		*D. magna*
LC50	3754	LC50	[illegible]	LC50	NA
95% UC	5364	95% UC	3668	NOEC	NA
95% LC	2626	95% LC	2830		
NOEC	1696	NOEC	1696		

Chronic Tests Results on Samples Collected from ESF in a WET Format (3 samples collected over 7-days)

C. triangulifer 14-day Growth Wt

Cond		
	IC25	[illegible]
	95% UC	1018.636
	95% LC	627.104
	NOEC	[illegible]
	LOEC	1268

C. dubia 7-day Fecundity

Test 1

Cond		
	IC25	3037.438
	95% UC	3569.509
	95% LC	895.077
	NOEC	3313
	LOEC	5116

Test 2

Cond		
	IC25	3895.922
	95% UC	4204.643
	95% LC	3432.734
	NOEC	3297
	LOEC	5614

Ex Situ ESF Continuous Flow Test – 20day Duration Chronic Results

Growth - Length

Cond		
	IC25	[illegible]
	95% UC	842
	95% LC	984
	NOEC	
	LOEC	567

Head Capsule Size

Cond		
	IC25	1343.7
	95% UC	1427
	95% LC	1292
	NOEC	807

Chronic Tests – Growth Results 7-day Larval Fathead Minnow From ESF

WET Test 1

Cond	IC25	[illegible]
	95% UC	4002.48
	95% LC	3327.72
	NOEC	
	LOEC	708
Ca	IC25	195.73
	95% UC	221.32
	95% LC	168.78
	NOEC	
	LOEC	12
Na	IC25	522.15
	95% UC	582.55
	95% LC	444.66
	NOEC	
	LOEC	32

WET Test 2

Cond	IC25	632.8
	95% UC	10859.95
	95% LC	520.58
	NOEC	
	LOEC	649
Ca	IC25	11.35
	95% UC	581.48
	95% LC	6.67
	NOEC	
	LOEC	12
Na	IC25	30.6
	95% UC	1562.45
	95% LC	19.82
	NOEC	
	LOEC	32

Ex Situ ESF Continuous Flow Test

Cond	IC25	[illegible]
	95% UC	8076.43
	95% LC	3350.58
	NOEC	5380
	LOEC	8800

Discussion

Culture
- EPA-ORD Cincinnati has successfully developed a defined culture method using known species of diatoms
- A survival rate >80% and a fecundity of 1000 eggs was achieved for five of seven generations
- The regression relationship between pre egg laying weight adults and fecundity was significant
- Regression analysis determined that a pre egg laying wet weight of >.00280g would give a 1000 egg count with a 95% confidence interval

Toxicity Comparisons

KCl
- In 48 hr tests, *C. triangulifer* was less sensitive to KCl than *C. dubia* and *D. magna*
- In Chronic Tests *C. triangulifer* was as sensitive as *C. dubia*

NaCl/CuSO4
- In 48 hr tests, *C. triangulifer* was over 2x more sensitive to NaCl and CuSO4 than *C. dubia* and *D. magna*
- In Chronic NaCl tests, *C. triangulifer* was 2.8X more sensitive than *C. dubia*

Preliminary Mesocosm Results
- Results of the 14-day Bench scale *C. triangulifer* Growth test are similar to the 20-day Ex Situ mesocosm study
- *C. triangulifer* were more sensitive than larval fathead minnows and *C. dubia* in both bench-scale (WET format) and Ex situ testing of mesocosm TDS doses
- Only the control stream showed full development of *C. triangulifer*, of the 60 individuals that started the test, 21 were nymphs, 29 with wing pads or sub imago - 10 may have already emerged as adults and were not found
- Larval Fathead results indicate larval fish may be as sensitive or close to *Ceriodaphnia* sensitivity
- Organic Material and/or Suspended Solids may have slight influence on toxicity of salts.

Future Research

C. triangulifer Testing
- Finish current Chronic testing of CuSO4
- Finish analyzing Total Dissolved Solids (TDS) test results from EPA's Experimental stream Mesocosm Study – Bench scale and in situ testing of 130 - 5500 mg/L
- Start assessing other resource extraction TDS composition from Mountain Top Mining leachates and other oil and gas extraction areas of the U.S.

Field Experiments
- Deployments at Mountain Top Valley Fill Sites

Note: Co-author Dave Buchwalter from North Carolina State University has 27,000 partial gene sequences and have cloned 12 genes of interest to date. For more information contact Dave at dbbuchwa@ncsu.edu