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Preliminary results of laboratory toxicity tests with the mayfly, *Isonychia bicolor* (Ephemeroptera: Isonychiidae) for development as a standard test organism for evaluating streams in the Appalachian coalfields of Virginia and West Virginia

Brandi Shontia Echols · Rebecca J. Currie · Donald S. Cherry

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Abstract Selecting the most appropriate test species for sediment and water column assays has been a primary goal for ecotoxicologists. Standard test organisms and established test guidelines exist, but the USEPA-recommended species may not be the most sensitive organisms to anthropogenic inputs. This paper describes preliminary results of toxicity tests with the mayfly, *Isonychia bicolor* (Ephemeroptera). Results suggested that *Isonychia* were moderately sensitive to NaCl after 96 h with an average LC_{50} value of 3.10 g NaCl per liter. This value decreased after 7 days of exposure, resulting in a mean LC_{50} value of 1.73 g NaCl per liter. When exposed to a coal-mine-processed effluent, *Isonychia* generated LC_{50} values that ranged from 13% to 39% effluent. *I. bicolor* were more sensitive to the coal processing effluent than *Ceriodaphnia dubia* with conductivity lowest observable effects concentration (LOEC) values for mayfly survivorship that ranged from 1,508 to 4,101 μS/cm, while LOEC values for *C. dubia* reproduction ranged from 2,132 to 4,240 μS/cm.

B. S. Echols (✉) · R. J. Currie · D. S. Cherry
Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, USA
e-mail: echols@vt.edu

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Introduction

The overall goal of aquatic ecotoxicology is the assessment and ultimate protection of aquatic ecosystems. This goal in part is accomplished through the process of risk assessment and the derivation of ambient water quality guidelines that provide a regulatory format for protecting ecosystem integrity and biodiversity. In order to develop appropriate guidelines, the development of toxicity tests that use test organisms that will provide the maximum amount of protection for naturally occurring species is necessary. Determining the appropriate test organism, organism age, and most sensitive test end points has been a challenging task for researchers and has lead to the acceptance of a battery of specific organisms outlined in current US Environmental Protection Agency (USEPA 2000, 2002a, b) regulatory guidelines. While organisms selected by the USEPA have proven to be useful in regulating the toxicity of effluents to receiving systems and monitoring ecosystem conditions, these may not always serve as appropriate surrogates for the protection of the most sensitive species within receiving systems.

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There are many benefits to utilizing these established standard test organisms, which include *Ceriodaphnia dubia*, *Daphnia magna*, *Pimephales promelas*, *Chironomus dilutus* (formerly *Chironomus tentans*), *Hyalella azteca*, and *Lumbriculus variegatus*. These organisms are relatively easy to culture, require a minimal amount of labor to use in tests, provide limited variability between individuals, and have been used extensively, following standard guidelines, so a large database of toxicological responses is available. Although results of tests with these species drive the derivation of water quality criteria and are used as required species in national pollutant discharge elimination system permits, they are not widely distributed in aquatic systems. Standard test organisms may not be representative of the most sensitive species found in specific habitats, making extrapolation of laboratory results to instream benthic assemblages difficult (Rosenberg and Resh 1996). Cherry et al. (2002) examined the acute toxicity of copper to 17 organisms for the development of site-specific criteria in the Clinch River, VA, USA. Results of this study were used to determine a ranking of sensitivities in which *C. dubia* and *P. promelas* ranked sixth and 14th, while the top four organisms included *Lampsilis*, *Medionidus*, *Villosa*, and *Isonychia*. Hence, *Isonychia* would be more protective to aquatic life against copper stress than the two USEPA test species, *C. dubia* and *P. promelas*.

There are numerous aquatic organisms that can be selected as test species, but finding the most appropriate organism that meets the necessary criteria is challenging. Suitable test species should be ecologically important, sensitive enough to a range of pollutants to provide relevant protection to aquatic habitats, easily cultured in the laboratory or collected in the field, and allow for the development of standard testing protocols with adequate control survivorship. A number of benthic macroinvertebrates have been suggested as standard toxicity tests species, but few of them have the attributes that qualify them as a possible indicator organism in laboratory tests. Due to the well-documented sensitivity of insects in the order Ephemeroptera (Pontasch and Cairns 1988; Short et al. 1991; Williams and Williams 1998; Hickey and Clements 1998; Clements et al. 2002) to both natural and anthropogenic stressors, some researchers (Kennedy et al. 2004) feel that the development of a standardized toxicity test using mayflies may be more beneficial for assessing potential adverse effects of point source discharges on aquatic organisms.

To date, one of the most common mayfly species used for toxicity testing has been the burrowing mayfly, *Hexagenia limbata*, which has been used extensively in sediment toxicity tests (Rand et al. 1995). The extension of such tests to include other genera of mayflies in water column toxicity tests has occurred intermittently over the past three decades (Sherberger et al. 1977; Peters et al. 1985; Diamond et al. 1992; Dobbs et al. 1994; Beketov 2004; Kennedy et al. 2004; Hassell et al. 2006; Brinkman and Johnston 2008; O'Halloran et al. 2008), with a variety of mayfly species and variable test designs. For example, Sherberger et al. (1977) examined the effects of thermal shock on *Isonychia sadleri* nymphs in laboratory simulations. Tests were conducted in preheated water baths by immersing test chambers (fiberglass boxes) into the water baths for a given time period then returning larvae to water baths maintained at acclimation temperature. Peters et al. (1985) determined responses of *Isonychia bicolor* nymphs exposed to alkaline pH in laboratory toxicity tests. Tests were conducted in 2.5-l glass beakers which were placed on stir plates to create flow. Nymphs were contained in fiberglass screen cages within each beaker, which were suspended in the water column above the stir bar so no damage or stress would occur to the *Isonychia*. Organisms for this test were collected from Sinking Creek, Newport, VA, USA, and acclimated for 24–48 h. Tests included in this study were not fed. Kennedy et al. (2004) used similar methodology and test apparatus to evaluate coal mining discharges high in total dissolved solids (TDS); however, these tests were fed ~6 ml of yeast–cerophyll (cereal leaves)–trout chow (YCT) and ground Tetra-min® daily. Although these studies varied in methodology, they all utilized *Isonychia* as the test organism. Studies by Diamond et al. (1992) and Brinkman and Johnston (2008) used species of Heptageniid mayflies (*Stenonema modestum* and *Rhithrogena* *hageni*, respectively) to evaluate acute toxicity issues, while Beketov (2004) used several genera of mayflies to determine sensitivity differences to ammonia, nitrite, and nitrate.

The Kennedy et al. (2004) study found that *Isonychia* exhibited a high dose-dependent response to specific conductivity, had greater ecological relevance compared to standard test organisms, and were overall more protective of sensitive biota; however, test results were not as consistent as those conducted with *C. dubia*. Still, the lowest observable effects concentration (LOEC) to conductivity from a coal mining effluent was 1,562 μS/cm for *Isonychia* and more than twice as high (3,730 μS/cm) for *C. dubia*, supporting the need to develop a more ecologically relevant toxicity test to protect streams and rivers from certain discharges. The objective of this research was to provide a preliminary assessment of *I. bicolor* as a toxicity test organism for protecting lotic systems affected by coal mining in the Appalachian coalfields of Virginia and West Virginia. In 1995, the USEPA Office of Surface Mining reported that acid mine drainage, or AMD, was the single greatest threat to water quality in the Appalachian Mountain region of the USA. In addition, the effects of AMD on benthic macroinvertebrates have been extensively documented (Smith and Frey 1971; Herricks and Cairns 1974; Armitage 1980; Rutherford and Mellow 1994; Cherry et al. 1995, 1997, 2001; Winterbourn and McDiffet 1996) and have shown that biodiversity is generally reduced in streams impacted by AMD. Specifically, Clements (1991) found *I. bicolor* to be highly sensitive to heavy metals. Although AMD is most often the stressor correlated to poor stream health in coal-mining-impacted streams, point source discharges high in TDS and associated conductivity are gaining concern for their role in limiting benthic communities. Future regulations for multiple constituent stressors such as TDS will require the use of more ecologically relevant test organisms. By evaluating the response of *I. bicolor* to a standard reference toxicant and an environmentally relevant stressor, comparisons could be made between this mayfly species and standard toxicity test organisms, such as *C. dubia*, which are currently used to protect these streams.

Materials and methods

Description of the test organisms

The order Ephemeroptera along with Plecoptera and Trichoptera represent some of the most sensitive aquatic macroinvertebrates. These benthic macroinvertebrates are widely used in field studies to evaluate the environmental effects of point and nonpoint source pollution (Barbour et al. 1999). Ephemeroptera are well documented as sensitive indicators of water quality (Pontasch and Cairns 1988; Short et al. 1991; Williams and Williams 1998) particularly to contaminants such as metals and ammonia (Peckarsky and Cook 1981; Leland et al. 1989; Clements 1994; Clements and Kiffney 1995; Hickey and Clements 1998; Hickey et al. 1999; Beketov 2004) and have been regarded as the most sensitive order of aquatic invertebrates. *Isonychia* (Ephemeroptera: Isonychiidae) nymphs were selected for this research because of their ecological relevance, wide distribution (Edmunds et al. 1976; Unzicker and Carlson 1982), and use in previous studies (Sherberger et al. 1977; Peters et al. 1985; Sibley and Kaushik 1991; Diamond et al. 1990; Dobbs et al. 1994; Kobuszewski and Perry 1994; Cherry et al. 2002; Kennedy et al. 2004; Cherry and Soucek 2006). They are commonly found in swiftly flowing waters of medium to large creeks and rivers with a predominant distribution in eastern North America (Canada and USA; Edmunds et al. 1976). The streamlined nymphs are regarded as "vigorous" swimmers and are commonly found in areas of tangled vegetation, leaves, or other debris in fast-moving water (Edmunds et al. 1976). The negatively phototrophic nymphs cling to the substrate facing the current which facilitates their unique feeding habits. *Isonychia* are one of four ephemeropteran genera in North America which are considered suspension or filter feeders (Kondratieff and Voshell 1984). Using setae on their forelegs, these mayflies filter fine suspended particles in the 0.1- to 0.7-μm size range (Wallace and O'Hop 1979) from the water column. Filter-feeding organisms are an integral part of an aquatic ecosystem and contribute to the overall structure and function by filtering fine suspended particles from the water column.

Isonychia mayflies are a bivoltine insect having two generations per year with an overwintering cohort that emerges in the spring and a smaller summer/fall cohort that develops and emerges quickly in late summer and early fall (Kondratieff and Voshell 1984). Edmunds et al. (1976) suggest that *Isonychia* occurring in warmer regions of the southeastern coastal plain may emerge throughout the year, while emergence may be limited to warmer months only for nymphs found in colder climates.

Isonychia toxicity tests

I. bicolor nymphs were collected from Sinking Creek, a fourth-order tributary in Newport, VA (Giles County), USA, a reference area used in this laboratory for more than 25 years and used as a collection source in previous studies (Peters et al. 1985; Snyder and Hendricks 1997; Cherry et al. 2002; Kennedy et al. 2004; Cherry and Soucek 2006). *Isonychia* were collected using D-frame dip nets (Wildco, 425-A40, 800 × 900-μm mesh), subsorted in plastic trays, and gently transferred into coolers filled with aerated Sinking Creek water (SCW) using BioQuip® soft-touch forceps. Organisms were acclimated to laboratory conditions for ~5 to 14 days, depending on water temperature at time of collection. If water temperature was less than 8°C, then mayflies were allowed to acclimate slowly to testing temperature for a minimum of 2 weeks (~14 days). When collection temperature was greater than 8°C, time of acclimation was dependent upon the degree difference between collection temperature and desired testing temperature. Temperature was increased by ≤2° C daily (Kennedy et al. 2004). During this acclimation period, *Isonychia* remained in the coolers (5 l) with SCW, to minimize the stress of additional handling/transfer, and aerated (approximately two bubbles per second) using standard aquarium aerators. Water was renewed daily by siphoning approximately 75% of the water and replenishing with unfiltered, temperature-adjusted SCW. Mesh screen was placed into the coolers to provide a substrate for the mayflies to cling to. Mayflies were initially fed a mixture of ground Tetra-min® flakes and YCT, sieved to <200 μm. Later tests utilized a food mixture of ground Purina® dog chow, cereal leaves, and green algae (*Pseudokirchneriella subcapitata*) sieved to <150 μm, based upon gut analysis and food preferences outlined in Wallace and O'Hop (1979).

A representative sample of organisms was retained from each collection for size determination. Determining the developmental stage of the test organism is important in understanding their response to stress; however, the use of instars, as used for other routinely used test organisms, is not accurate for mayflies. Instar number can vary within species, even when reared in similar conditions (Brittain 1976, 1982; Clifford et al. 1979). Overall estimates of the number of nymphal instars are between 15 and 25 (Fink 1980). Therefore, the use of a morphological measurement can be used to indicate size class or stage of development. The subset of mayflies used for size determination was preserved in ~70% ETOH and measured for body length (mm) using an ocular micrometer on a Zeiss stereomicroscope (nearest 0.1 mm). Since toxicity tests took place during early spring and summer months, size class/developmental stages varied between toxicity tests. Organisms used for testing during early spring (January–February) ranged from ~9 to ~14 mm, while those used during late spring (April) were ~12 to ~18 mm in length. Mayflies used in summer testing (July) were much smaller (~5 to ~9 mm) in length.

Isonychia bioassays were conducted in 600-ml glass beakers containing a minimum of 500 ml of test solution or control water. Each beaker contained a 10.2 × 5-cm mesh screen, which served as a substrate for the mayflies. To provide flow and maintain DO_2 saturation, beakers were aerated (approximately two bubbles per second). Air stones were used to gently agitate the surface water, creating water column flow. Chronic toxicity tests were conducted with sodium chloride (NaCl, American-Chemical-Society-certified), a common salt used as a reference toxicant. Use of NaCl allowed results to be compared to a database of test results with other aquatic organisms. In addition, a coal mine processing impoundment (CPI) effluent obtained from the Callahan Creek Watershed (CCW) in Wise Co., VA, USA, with moderately high conductivity and TDS, was used to further test the response of *Isonychia* under realistic conditions. Results of these tests were compared to responses of *C. dubia*, also exposed to the coal processing effluent.

Ceriodaphnia dubia toxicity tests

C. dubia were used in chronic toxicity tests to determine the comparative sensitivity of this daphnid to *Isonychia* mayflies. *Ceriodaphnia* 7-day chronic toxicity tests were conducted using the same CPI effluent obtained from the CCW, following USEPA (2002b) protocols. Daphnids (<24 h old) were isolated from cultures that were less than 14 days old and that had <20% adult mortality. Bioassays were conducted in 50-ml glass beakers with a minimum of 40-ml test solution. Test concentrations ranged from 6.25% to 100% effluent, consisting of ten replicates per concentration, with one daphnid per replicate. Control and diluent water used for testing were moderately hard synthetic freshwater. Test concentrations were renewed daily. Daphnid survivorship and neonate production were also observed and recorded daily, and organisms were fed a mixture (0.40/50 ml) of green algae (*P. subcapitata*) and YCT. Water chemistry was measured daily on renewal (in) and outgoing (out) water for temperature (°C), pH (standard units), conductivity (μS/cm), and dissolved oxygen (DO_2, mg/l). Alkalinity and hardness (mg $CaCO_3$/l) were also measured on renewal water for the highest and lowest (control) concentrations.

Trace metal analysis of CPI effluent

Trace metals known for toxic consequences were analyzed by induced coupled plasma–mass spectrophotometry (ICP–MS) from the CPI effluent. Dissolved metals in the effluent included arsenic, cadmium, cobalt, chromium, copper, iron, lead, mercury, nickel, selenium, and zinc along with benign trace elements such as calcium, magnesium, potassium, sodium, and strontium.

Statistical analysis

Toxicity test end points (survivorship and reproduction for daphnids only) were analyzed with TOXSTAT® (3.3/1996, University of Wyoming Department of Zoology and Physiology, Laramie, WY, USA) using appropriate parametric (Dunnett's test) and nonparametric (Steels–many one rank test) procedures ($\alpha = 0.05$). Lethal concentration values (LC_{50}) were determined using trimmed Spearman–Karber method (Hamilton et al. 1977). Correlation analyses were performed using JMP IN (7.0.2/2008, SAS, Cary, NC, USA).

Results and discussion

Isonychia reference tests with sodium chloride

Mayfly survivorship was observed every 24 h, and LC_{50} values were determined at 48, 72, and 96 h and at the end of 7 days of exposure to NaCl (Fig. 1). All four tests were similar at 48 h with LC_{50} values >8.00 g/l NaCl. After 72 h, LC_{50}

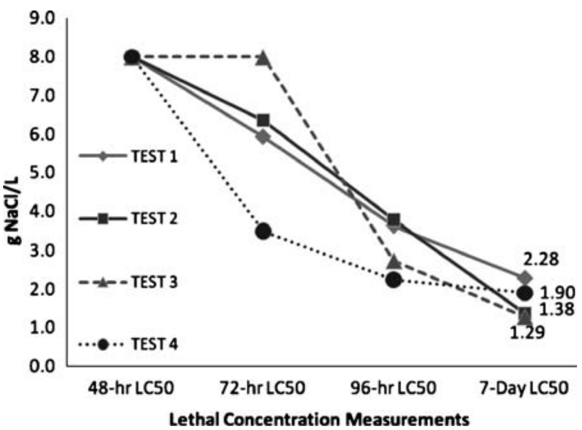

Fig. 1 Lethal concentration (LC_{50}) values during a 7-day test period for *I. bicolor* exposed to NaCl

values ranged from 3.50 to >8.00 g/l NaCl with tests 1 and 2 being the most similar. Tests 3 and 4 had the highest (8.0 g/l) and lowest (3.5 g/l) LC_{50} values. This wide range of toxicity values may be attributed to the varying age and developmental stage of the mayflies. *Isonychia* used in tests 1, 2, and 3 were collected in January 2007 (test 1) and February 2008 (tests 2 and 3) during which they were several months away from their first emergence (May). Mayflies from test 4, which resulted in an LC_{50} value of 3.50 g/l after 72 h, were in the latter stages of development having been collected in April 2008. Unfortunately, methods for determining specific instars for mayfly nymphs are unreliable (Fink 1982); therefore, instars were not used in determining organism age or stage of development. After 4 days of exposure (96 h), *Isonychia* LC_{50} values began to decrease substantially, ranging from 2.25 to 3.78 g/l NaCl with a mean LC_{50} of 3.10 g/l. Test 3 had the greatest change in mortality from the 72-h interval to the 96-h interval (Fig. 1). After 7 days, values ranged from 1.29 to 2.28 g/l NaCl with a mean LC_{50} value of 1.73 g/l NaCl.

Minimal control mortality was observed with ≥80% of the mayflies surviving through 7 days of exposure for each of the four reference (NaCl) tests (Fig. 2). Values for conductivity in the lowest concentration of NaCl tested ranged from 1,147 to 1,878 μs/cm (mean = 1,373 μS/cm) and had ≥60% survival at test termination. The highest conductivity tested ranged from 14,240 to 14,270 μS/cm (mean = 14,247 μS/cm) in tests 1, 2, and 3 and resulted in 100% mortality of test organisms by test day 6. Test 4 had similar results with high conductivity of 12,000 μs/cm, which resulted in 100% mortality by day 6. The second highest conductivity values were 7,370, 7,200, 7,411, and 6,460 μS/cm in tests 1, 2, 3, and 4, respectively. These values for conductivity were the highest tested that did not result in 100% mortality in any of the four tests by test termination. Mayfly survival was negatively correlated with conductivity in all four reference tests (Fig. 2).

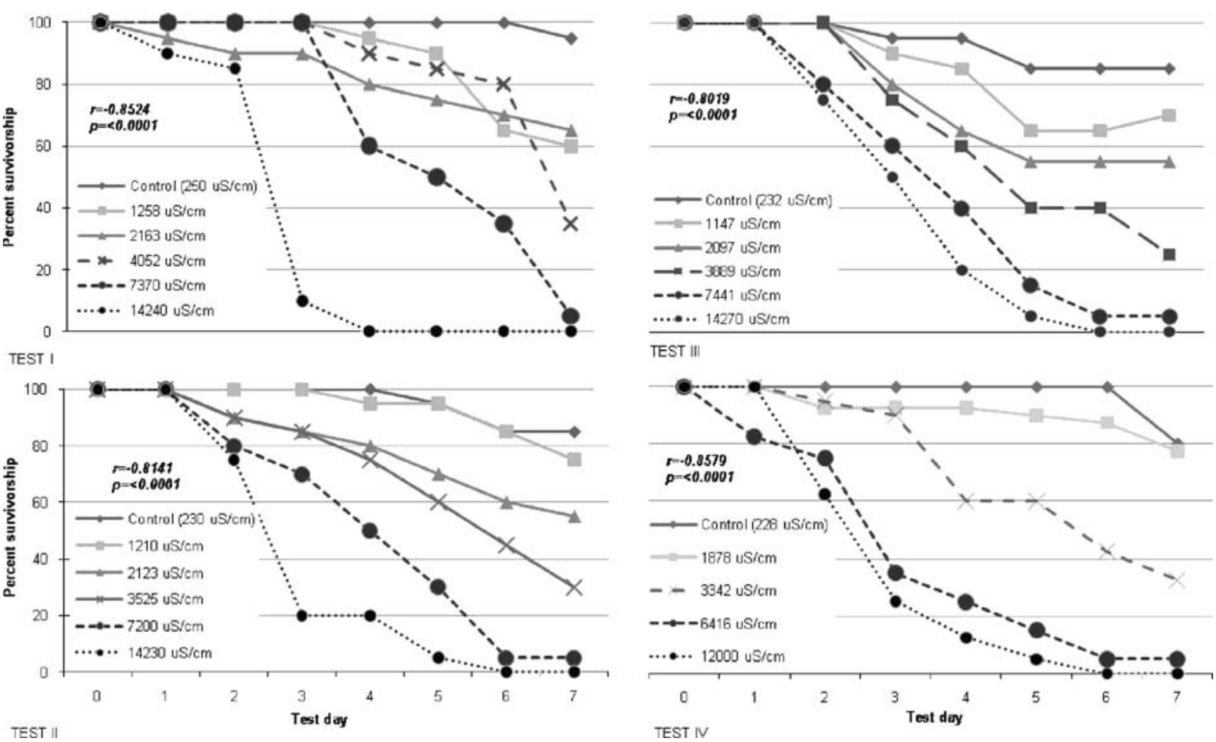

Fig. 2 *I. bicolor* survivorship in 7-day NaCl chronic toxicity tests. *Dashed lines* indicate concentrations which are significantly different from the control

Table 1 Comparison of test organism lethal concentration (LC_{50}) values for sodium chloride (g NaCl/l) at 24, 48, 72, and 96 h

Test organism		LC_{50} values (g NaCl/l)				Citation
Taxonomic group	Genera	24-h mean (range)	48-h mean (range)	72-h mean (range)	96-h mean (range)	
Cladocera: Daphniidae	*Ceriodaphnia dubia*	3.38 (3.08–3.54)	1.96 (1.77–2.33)	–	–	Mount et al. (1997)
Cladocera: Daphniidae	*Daphnia pulex*	–	2.26 (1.47–3.05)	–	–	Birge et al. (1985)
Ephemeroptera: Heptageniidae	*Stenonema rubrum*	–	2.50 (2.50–2.50)	–	–	Roback (1965)
Cladocera: Daphniidae	*Daphnia magna*	6.38 (6.38–6.38)	4.88 (3.31–6.03)	–	–	Dowden and Bennett (1965); Hoke et al. (1992); Harris (1994); Arambasic et al. (1995); Mount et al. (1997)
Gastropoda: Physidae	*Physa heterostropha*	5.53 (4.20–7.5)	5.13 (3.70–6.95)	4.89 (3.5–6.2)	4.72 (3.5–6.20)	Wurtz and Bridges (1961)
Diptera: Chironomidae	*Cricotopus trifasciatus*	–	6.22 (6.22–6.22)	–	–	Hamilton et al. (1975)
Tricoptera: Hydroptilidae	*Hydroptila angusta*	–	6.62 (6.62–6.62)	–	–	Hamilton et al. (1975)
Ephemeroptera: Ameletidae	*Ameletus* sp.	> 8.00	6.96 (5.91–8.0)	5.14 (4.07–6.20)	4.13 (3.25–5.01)	Echols (unpublished data)
Cypriniformes: Cyprinidae	*Pimephales promelas*	7.92 (7.1–9.0)	7.69 (7.05–8.7)	7.65 (7.65–7.65)	7.62 (7.62–7.62)	Adelman and Smith (1976)
Diptera: Chironomidae	*Chironomus attenuatus*	9.82 (9.82–9.82)	7.99 (7.99–7.99)	–	–	Thornton and Sauer (1972)
Ephemeroptera: Isonychiidae	*Isonychia bicolor*	> 8.00	> 8.00	5.95 (3.50–8.00)	3.10 (2.25–3.78)	–
Amphipoda: Hyalellidae	*Hyalella azteca*	–	–	–	6.51 (6.51–6.51)	Lasier et al. (1997)
Tricoptera: Hydropsychidae	*Hydropsyche* sp.	–	–	–	9.00 (9.00–9.00)	Roback (1965)
Diptera: Culicidae	*Culex* sp.	10.50 (10.50–10.50)	10.20 (10.20–10.20)	–	–	Dowden and Bennett (1965)
Odonata: Coenagrionidae	*Argia* sp.	32 (32–32)	29 (26–32)	25 (24–26)	23.5 (23–24)	Wurtz and Bridges (1961)

Isonychia sensitivity to NaCl was also compared to 15 additional aquatic test organisms (Table 1). This comparison included three routinely tested daphnids, the fathead minnow (*P. promelas*), an amphipod (*H. azteca*), a freshwater snail (*Physa heterostropha*), and eight benthic macroinvertebrates, including two additional mayflies in the families Heptageniidae and Ameletidae. Data were obtained from the PAN Pesticides Database (http://pesticideinfo.org/2008) and consisted of previously published LC_{50} values for each species. LC_{50} values were averaged for 24, 48, 72, and 96 h, where applicable. Species sensitivity to NaCl ranged from 3.38 g/l for the daphnid (*C. dubia*) to 32 g/l for the damselfly (*Argia* sp.) after 24 h of exposure. *Isonychia* and *Ameletus* mayflies had similar LC_{50} values (>8 g/l) after 24 h.

Many of the organisms included in Table 1 were only tested for 48 h which represents the exposure duration for many acute tests. Mayfly sensitivity to NaCl increased after 48 h, so data generated after 48 h were compared to 72- and 96-h LC_{50} values for *Physa*, *Ameletus*, fathead minnows, *Hyalella*, *Hydropsyche*, and *Argia*. At 72 h, LC_{50} values generated for *Isonychia* were lower than those for fathead minnows and *Argia*, and after 96 h *Isonychia* had the lowest LC_{50} value at 3.10 g NaCl per liter, half the reported value for *H. azteca* and a third of the LC_{50} value reported for *Hydropsyche* sp. Organisms in the family Hydropsychidae, particularly *Hydropsyche* sp., have been reported to have a high tolerance to salinity (Williams and Williams 1998; Blinn and Ruiter 2006; Kennedy et al. 2003).

A comparison of alkaline pH exposure between *Isonychia* (96-h LC_{50}) and *Ceriodaphnia* (48-h LC_{50}) indicated that both test organisms had the same tolerance, with an LC_{50} value of 10.3 g NaCl per liter in static laboratory tests (Peters et al. 1985; Belanger and Cherry 1996). However, when *Isonychia* were tested in a continuously flowing artificial stream system receiving New River (VA,

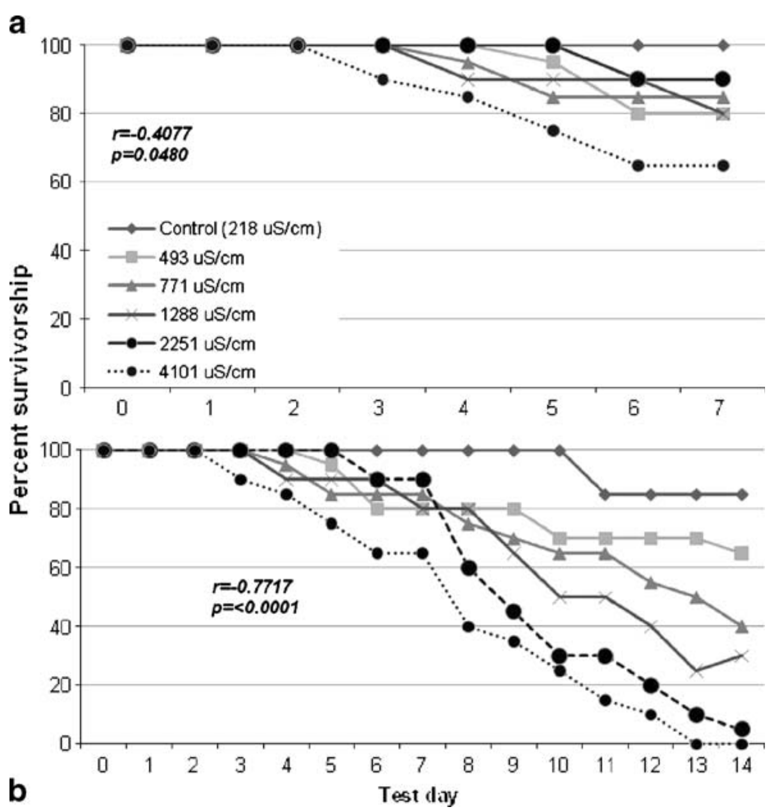

Fig. 3 *I. bicolor* chronic test 1 with CCW CPI effluent over 7 days (**a**) and 14 days (**b**). *Dashed lines* indicate concentrations which are significantly different from the control

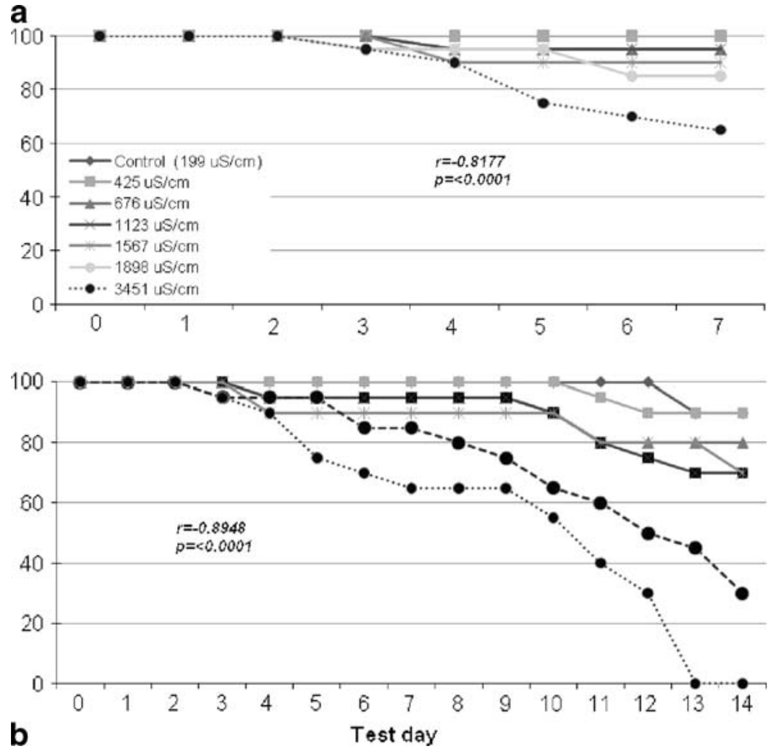

Fig. 4 *I. bicolor* chronic test 2 with CCW CPI effluent over 7 days (**a**) and 14 days (**b**). *Dashed lines* indicate concentrations which are significantly different from the control

USA) water, the 96-h LC_{50} value declined to 9.5 g NaCl per liter under more environmentally realistic conditions for the mayfly. Therefore, mayflies appear to be more sensitive to alkaline pH than *C. dubia*.

Isonychia bicolor CCW CPI effluent tests

Data from CCW CPI effluent tests with *Isonychia* were analyzed at 7 and 14 days to determine differences in mayfly response during the duration of testing and for comparison with other test organisms (Figs. 3, 4, and 5). Conductivity in test 1 ranged from 493 to 4,101 μS/cm, and 7-day results showed a poor correlation between survival and conductivity ($r = -0.4077$) that improved by the end of the 14-day exposure period ($r = -0.7717$; Fig. 3). In test 2, a stronger relationship was observed between survival and conductivity after 7 days ($r = -0.8177$) that increased after an ad-

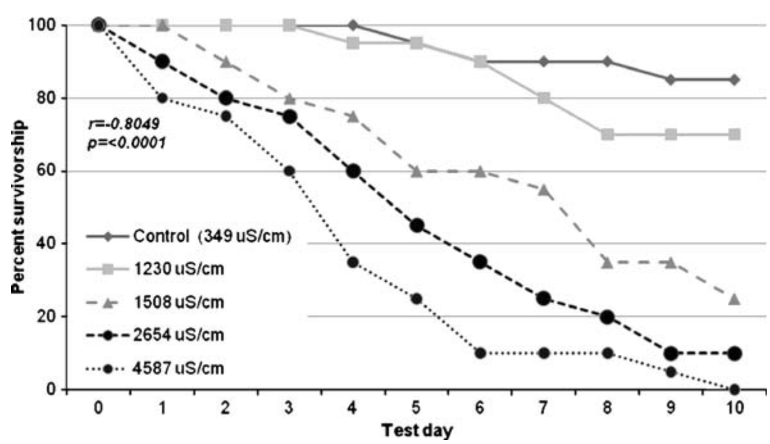

Fig. 5 *I. bicolor* chronic test 3 with CCW CPI effluent over a 10-day test period. *Dashed lines* indicate concentrations which are significantly different from the control

ditional 7 days of exposure ($r = -0.8948$; Fig. 4). Test 3 was conducted for 10 days with a strong negative correlation between mayfly survival and conductivity ($r = -0.8049$; Fig. 5). In each of the three tests, 100% mortality occurred in the highest test concentrations (>4,000 μS/cm) by test termination. Calculated LC_{50} values for the CCW CPI effluent ranged from 13% to 39% effluent.

In tests 1 and 2 (Figs. 3 and 4), impairment was not observed until after 48 h of exposure to CCW CPI effluent, while mortality occurred in test 3 after the first 24 h (Fig. 5). Test 3 mayflies were collected from the summer cohort in July 2006, which may explain the more sensitive response seen in these organisms. Developing standard guidelines including standard collection methods, holding conditions and acclimation, optimal size class of mayflies, and overall general condition of the organisms will help to reduce variability in sensitivity that may occur.

Five chronic tests were conducted with *Ceriodaphnia* exposed to the CPI effluent. Percent survival in 100% effluent ranged from 0% to 100% (Table 2). Significant impairment was observed in two of the five tests with LOEC values for reproduction reported at 75% and 50% effluent for tests 3 and 4, respectively. Mortality in 100% effluent and reproductive impairment were not correlated with conductivity values. Test 4 had varying mortality in 100% effluent and reduced reproduction, but the effective values for conductivity were similar to these reported in the other four tests.

Table 2 *I. bicolor* survivorship and *C. dubia* survivorship and reproductive responses in 7-day chronic toxicity tests with CCW CPI effluent

Test number	Percent survivorship 100% effluent	Survival/ reprod. LOEC	Mean conductivity for LOEC (μS/cm)
I. bicolor			
Test 1	65	100	4,101
Test 2	65	100	3,451
Test 3	10	25	1,508
C. dubia			
Test 1	80	100	4,250
Test 2[a]	100	100	4,014
Test 3	100	75	3,132
Test 4	0	50	2,132
Test 5[a]	90	100	3,403

[a]Test concentrations were based on conductivity range and not serial dilution

Table 3 Trace metals and other elements measured in the coal processing impoundment effluent in test 1

Parameter	Result	Detection limit (μg/l)
Arsenic	ND	10.0
Cadmium	ND	2.0
Cobalt	ND	7.0
Chromium	ND	5.0
Copper	ND	25.0
Iron	ND	100.0
Lead	ND	3.0
Mercury	ND	0.20
Nickel	ND	40.0
Selenium	8.5	5.0
Zinc	ND	20.0
Other trace elements		
Calcium	68,800	5,000
Magnesium	50,700	5,000
Potassium	31,800	5,000
Sodium	957,000	25,000
Strontium	7,020	50.0

Significant reductions for mayfly survival and *Ceriodaphnia* reproduction in 100% CPI effluent were considered to be primarily from the ionic salts in the TDS and not due to trace metal influence (Table 3). In test 1, the CPI 100% effluent had conductivity >4,000 μS/cm and TDS of 2,900 mg/l, but ten of the 11 trace metals analyzed had results that were equal to or lower than nondetectable limits (Table 3). Other than iron (100 μg/l), most detection limits were ≤40 μg/l (Ni) to 0.20 μg/l (Hg), with most of them being between 7 and 20 μg/l. The TDS comprising the effluent basically came from strontium, magnesium, potassium, calcium, and sodium with concentrations that ranged from 7,020 to 957,000 μg/l.

Summary and conclusions

Isonychia were moderately sensitive to NaCl, and after 7 days, LC_{50} values ranged from 1.29 to 2.28 g/l NaCl which is comparable to *C. dubia* sensitivity at 48 h. When compared to other organisms at 96 h, *I. bicolor* was more sensitive than *H.*

azteca or *P. promelas*, both considered standard organisms for routine toxicity testing.

Our toxicity testing results indicated that *Isonychia* were substantially more sensitive to the CCW CPI mining effluent than the 7-day *Ceriodaphnia* test, although the mayfly tests lasted 10–14 days. The LOEC for mayfly 10–14-day survivorship ranged from 1,429 to 2,251 μS/cm relative to the *Ceriodaphnia* results (∼4,000 μS/cm). These trends were similar (1,562 μS/cm for *Isonychia* and 3,730 μS/cm for *C. dubia*) to those reported by Kennedy et al. (2004) when testing a coal mining effluent from Leading Creek, OH, USA. Since the CCW CPI effluent had minimal trace metals present with concentrations below water quality criteria or detection limits, the suspected factor influencing mayfly toxicity was likely ionic salts. This has implications for the establishment of water quality criteria and discharge limits in the coal fields of Virginia and West Virginia, particularly limits for TDS. For the CCW CPI effluent, which is comprised of nontoxic trace elements and very low trace metal concentrations, impairment levels of TDS may occur at ∼1,400 mg/l. Distinguishing between trace metal toxicity and TDS toxicity will require additional testing with *Isonychia* and perhaps other relevant test organisms to determine the most appropriate upper TDS level.

An update to the Water Quality Standards in Alaska was recently proposed with a recommendation for revision of the TDS limit from 500 to 1,500 mg/l based upon a site-specific criterion (SSC) developed in Red Dog Creek (http://www.dec.state.ak.us/water/wqsar/wqs/pdfs/reddogfactsheet092605.pdf, 2005). The higher SSC proposed for the TDS limit was based upon a study by Chapman et al. (2000), whereby they found no toxicity to embryos or fry of rainbow trout, at greater than 2,000 mg/l TDS. They also emphasized that TDS toxicity was influenced by ionic content of the test solution especially that of mining effluents. The authors also found that chironomid larvae exposed to an artificially prepared effluent did not exhibit any toxicological response at 1,134 mg/l but had reduced growth (45% reduction in dry weight) at 2,089 mg/l. A second artificial effluent generated reduced survival at 1,750 and 2,240 mg/l, but no effects were observed at 1,220 mg/l TDS. Revising the existing TDS standard of 500 to 1,500 mg/l is a substantial increase.

The results of this research bring to light several advantages of using *I. bicolor* to evaluate toxicity resulting from coal-mining-influenced discharges to receiving systems, particularly in the Appalachian region of Virginia and West Virginia. *Isonychia* mayflies were more sensitive to a coal mining effluent than the current widely used test species, *C. dubia*, and to the reference toxicant, NaCl. Few mayflies in the families Ephemerellidae and Heptageniidae were collected in the Callahan Creek (VA, USA) watershed where mining activities are ongoing, although *Isonychia* were present, supporting the use of a resident species to protect the receiving system. According to Lowe and Butt (2007), for ecological assessments, many researchers evaluating soil toxicity are increasingly using ecological relevant species that are site specific. Hull et al. (2006) assessed the use of transplanted Asian clams (*Corbicula fluminea*) in the Clinch River to predict adverse effects on resident bivalves. In addition, preexisting toxicity data are available for *Isonychia* and other genera of mayflies contributing to a growing database of mayfly sensitivity.

However, *Isonychia* are thought to be a more tolerant mayfly genera than some other types found in Central Appalachia streams (e.g., ephemerellids, heptageniids) according to Pond et al. (2008). Additional mayflies critiqued for their use in toxicity tests due to increased tolerance included *Hexagenia*, *Centroptilum*, and *Cloeon*. Studies have demonstrated that mayflies may exhibit significant differences in sensitivity to toxicants not only between families and genera, but also between species within the same genus. Beketov (2004), researching the sensitivities of mayflies to ammonia, nitrite, and nitrate, reported that *Baetis vernus* was more sensitive than *Baetis fuscatus*. Handling stress in field-collected mayflies and laboratory acclimation are also areas that need further research. Laboratory culturing of *Isonychia* has only been attempted on a limited scale. Some limitations to culturing occur from the lotic habitat preference of these organisms and the need for flowing water which requires an examination of flow and dissolved oxygen requirements.

Choosing the most sensitive mayfly would provide the most conservative data, but the most sensitive species are not always well suited for laboratory testing. The goal in establishing a mayfly bioassay is to provide predictive data that can be used to protect the integrity of aquatic systems, but the chosen species must be reliable enough for repetitive testing in multiple laboratories; handling stress must be tolerated, and control survival must meet a specific criteria. Control survivorship varies among test organisms, but generally >80% survivorship is deemed acceptable and is typically recommended for routine toxicity tests.

Although the results of this study provide a preliminary assessment of *I. bicolor* use in toxicity testing, further research must be conducted to examine these organisms across varying life stages and develop valid sublethal end points for chronic testing. Head capsule width and body length and weight may prove useful in assessing impairment whereas molting may be too sensitive of an end point to be predictive. In addition, an attempt to establish laboratory culturing techniques for *Isonychia* would further help to establish the ability to utilize these organisms in multiple laboratories.

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