Ecotoxicity study for mayflies exposed to ambient stream water from the upper Delaware River Basin, reference toxicant, and to produced-water from natural gas drilling

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This was an ecotoxicity study that examined ambient water and water produced from natural gas drilling (i.e., water coming from a natural gas well after the well was hydrofracked, and gas collection has begun). Its purpose was to measure responses, if any, of benthic macroinvertebrates (in our study, mayflies) that are common and important components of stream ecosystems, and compare these responses to those of standard lab organisms (i.e., the water flea *Ceriodaphnia dubia* and the fathead minnow *Pimephales promelas*) commonly used to assess toxicity and set effluent limits. Benthic macroinvertebrates were chosen as a focus of this study because they are an ecologically important group of aquatic organisms that are commonly included in water quality assessment programs (Hellawell 1986, Resh 2008), and because they have provided water quality assessment programs with valuable insight for more than 100 years (Cairns and Pratt 1993). The presence or conspicuous absence of certain macroinvertebrate species at a site is a meaningful record of environmental conditions during the recent past, including ephemeral events that might be missed by assessment programs that rely on periodic water chemistry samples (Weber 1973, Barbour et al. 1999). Among the aquatic macroinvertebrates, mayflies were chosen for our study because they are known to be relatively sensitive to changes in water quality and play an important role in the commonly used EPT Index (Lenat and Penrose 1996).

Experimental Design and Methods

Water Chemistry

Water chemistry parameters were analyzed for the initial ambient and produced-water by the New Jersey State Department of Health and Senior Services, Public Health and Environmental Laboratories, Environmental and Chemical Laboratory Services located in Trenton, NJ. At the termination of the whole life tests pH, temperature, conductivity, dissolved oxygen were anaylyzed at SWRC and ammonia, hardness and alkalinity were determined at the lab in Trenton.

Study Species

Baetidae: *Centroptilum triangulifer*

Centroptilum triangulifer is a parthenogenetic (clonal) mayfly species (Funk et al. 2006) that is most abundant during summers when it has a relatively rapid development (25-30 d at 20°C). The primary clone we have worked with in these studies (i.e., Stroud Water Research Center [SWRC] Clone WCC-2®) occurs in low numbers during the winter, with minimal growth below 10°C. The WCC-2 was initially obtained from White Clay Creek, a piedmont stream of moderately high hardness located in southeastern PA. This stream has been designated Exceptional Value or defined as the “best” cold-water fisheries by PA Department of Environmental Protection. We find *C. triangulifer* commonly in the along the edges and slow current areas of 3^{rd} – 4^{th} order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from 19 states and provinces ranging from Quebec to Florida in the east and from Iowa to Texas in the west. This species been used for toxicity studies by Stroud since the early 1990s (e.g., Sweeney et al, 1993). Parthenogenetic reproduction (Funk et al. 2006) has allowed us to maintain cultures of a specific clone (WCC-2) of *C. triangulifer* in the laboratory almost continuously since the late 1980s, without risk of inbreeding or other genetic changes. This specific clone has also been recently used in a number of experiments examining the effects of thermal changes and extremes (SWRC unpublished data), elevated phosphorus (SWRC unpublished data), and the toxic effects of cadmium, mercury, selenium and zinc (Conley et al. 2009; Xie et al. 2009; Xie et al. 2010; Conley et al. 2011, Xie and Buchwalter 2011; Kim et al. 2012) and various ions in solution (David B. Buchwalter, NC State University, unpublished data; James Lazorchak, USEPA Cincinnati, personal communication).

Baetidae: *Procloeon rivulare*

Procloeon rivulare is a sexual mayfly species that exhibits a life history similar to that of *C. triangulifer* except that it has a winter egg diapause. It is most abundant during summers when it has a relatively rapid development (25-30 d at 20°C). We find it along the edges and slow current areas of 1^{st} – 4^{th} order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from 14 states and provinces ranging from Nova Scotia to Mississippi in the east and from Indiana to Texas in the west. We have been working with *P. rivulare* in the laboratory since 2004, collecting larvae from White Clay Creek as needed. We have not maintained a culture in the laboratory because of the risk of inbreeding and other genetic changes.

Baetidae: *Pseudocloeon frondale*

Pseudocloeon frondale is a sexual mayfly species that exhibits a life history similar to that of *C. triangulifer*, but like *P. rivulare*, it has a winter egg diapause. It is most abundant during summers when it has a relatively rapid development (28-31 d at 20°C). We find it along the edges and slow current areas of 3^{rd} – 4^{th} order streams in the mid-Atlantic region of the United States. Literature and online sources plus our own collections include records from 9 states and ranging from Maine to Florida west to Minnesota and Texas. We have been working with *Ps. frondale* in the laboratory since 2004, collecting larvae from White Clay Creek as needed. We have not maintained a culture in the laboratory because of the risk of inbreeding and other genetic changes.

Experiment 1: Whole-lifecycle (chronic) toxicity tests for ambient stream water

This experiment was designed as a "proof of concept", addressing the question: Does mayfly survivorship differ among streams believed to have different water qualities. We worked with three mayfly species (*C. triangulifer*, *P. rivulare*, *Ps. frondale*) and 5 water sources (Table 1):

Controls

- Dyberry Creek (41.661389°N, -75.288056°W) – Exceptional Value stream with soft water sampled above intersection of State Route 4007 and Sr4017
- White Clay Creek (39.859298°N,-75.783746°W) – Exceptional Value stream with moderately hard water sampled above Spencer Road at the SWRC

Upper Delaware River Test Sites

- Delaware River near Callicoon (41.756680°N, -75.057270°W) sampled on River Road below Bridge Street and the town of Callicoon
- Lackawaxen River near Honesdale (41.549938°N, -75.237223°W) sampled below Waste Water Treatment Plant on Bucks Cove Road

West Branch Lackawaxen River (41.673440°N, -75.376060°W) sampled below Route 247 (Creamton Drive)

Water parameters for these sites are presented in Table 2 (initial conditions) and Appendix 1 (final conditions). These were static (no renewal) tests with 30-60 day exposures beginning with newly hatched 1st instar larvae that were reared to the adult stage; the duration depended on the species and temperature (20°C). Four replicate tests with 50 individuals each were conducted with each of the 5 water types. Food (algae on plates) was provided as needed. Response variables that could be measured were survivorship (%), development time (days), and final body size (mg dry mass).

To evaluate the consistency of performance of the three mayfly species across tests (ambient vs. RT and produced-water), a two-way ANOVA was performed on survivorship, adult body size, development time, instantaneous growth rate (IGR), and population growth rate (PGR). A Tukey post hoc test was used for pairwise comparisons.

Experiment 2: Acute Reference Toxicant (RT) Tests – mayfly responses to NaCl

Experiment 2 was designed to quantify acute responses of *C. triangulifer* in short-term exposures to NaCl, and compare these results to data for two other mayfly species (i.e., *P. rivulare* and *Ps. frondale*), for *C. triangulifer* recently generated by EPA, and for two standard test species, *Ceriodaphnia dubia* and *Pimephales promelas* (data provided by Christoper J. Nally from the American Aquatic Testing, Inc. located in Allentown, PA). NaCl was chosen because it is a standard reference toxicant, and Na and Cl are major ion components in produced water. We conducted 24 acute RT tests (each test had two replicates of 20 individuals each; Table 1) that included two dilution waters (Dyberry and White Clay Creeks, for which the relationship between conductivity and NaCl concentration is shown in Figure 1), and two size classes (newly hatched 1st instar and middle instar) for *C. triangulifer* (to assess the significance of larval size in acute responses) and one size class (newly hatched 1st instar larvae) for *P. rivulare* and *Ps. frondale* (to assess comparability of *C. triangulifer* relative to other mayfly species). Each replicate had six treatments: control (Dyberry or White Clay Creek water) and five dilution concentrations [0 mg NaCl/L in Dyberry or White Clay Creek water, 206, 412, 824, 1647, 3295 (first instars only, all three species), 6590 (middle instar *C. triangulifer* only)]. These were static (no renewal) experiments, conducted at 20°C for 48 h. 20µL of a diatom slurry was provided as food in each test vessel. Response assessed was survivorship reported as concentration of NaCl associated with 50% mortality (LC50) of test population from that observed under control conditions using the Probit model (SAS Institute Inc., Cary, NC, version 9.3). For comparability with produced-water, these RT dilutions and LC50s were also expressed as specific conductance (a common measure of dissolved ion concentrations) and mg Cl/L (chloride is a known, often regulated toxin in fresh water ecosystems).

Experiment 3: Chronic Reference Toxicant (RT) Tests – chronic (whole lifecycle) mayfly responses to NaCl

Experiment 3 was designed to quantify chronic responses of *C. triangulifer* in whole lifecycle exposures to NaCl, and compare these results to data for *C. triangulifer* recently generated by EPA, and for two standard test species (*C. dubia* and *P. promelas*). NaCl was chosen because it is a standard reference toxicant, and Na and Cl are major ion components in produced water. We conducted two chronic RT tests with *C. triangulifer*, one using Dyberry Creek water and one using White Clay Creek water (Table 1). These were whole-life tests that began with the introduction of 50 newly hatched first instar larvae into each 1.9-L jars filled with water (static, no renewal) and an air stone to maintain oxygen saturation. Larvae were fed algae grown on acrylic plates, replenished as needed. For each test there were 6 treatments (0, 206, 412, 824, 1647, and 3295 mg/L added NaCl) with four replicate jars per treatment. Tests were run at 20°C until all remaining larvae had emerged as adults (48 days). Emerging adults were trapped in cages over the jars and collected daily. Basic water chemistry data for these tests are presented in Appendix 2.

Chronic response variables that could be measured were survivorship (%), development time (days), final body size (mg dry mass), instantaneous growth rate (IGR), and population growth rate (PGR). IGR reflects the growth (biomass) that occurs per day from 1st instar to adult and is calculated as: $\ln(W_f/W_i)$/development time in days, where W_f = final adult dry mass in mg (gravid weight in the case of females) and W_i = 0.0009 mg (hatchling mass). PGR is expressed as the number of eggs produced per individual (present at the start of the experiment) per day, calculated as: (fecundity x survivorship)/development time. This value is halved for sexual species (assuming half the population is female). Hypothesis tests (ANOVA with Tukey post hoc test) were used to determine the no observed effect concentration (NOEC or the highest concentration that did not differ from the control), and lowest observed effect concentration (LOEC or lowest test concentration that differed from control). For point estimation endpoints, Probit was used for LC50 and regressions were used to estimate IC25 (concentration needed to result in a 25% change in a response variable relative to the control).

Experiment 4: Acute toxicity tests of produced-water

Experiment 4 was designed to quantify acute responses of *C. triangulifer* in short-term exposures to a composite sample of produced water, and compare these results to data for two other mayfly species (i.e., *P. rivulare* and *Ps. frondale*), and for two standard test species (*Ceriodaphnia dubia* and *Pimephales promelas*). We conducted 16 acute toxicity tests (each test two replicates of 20 individuals each; Table 1) that included two dilution waters (Dyberry and White Clay Creeks), and two size classes (newly hatched 1st instar and middle instar) for *C. triangulifer* (to assess the significance of larval size in acute responses) and one size class (newly hatched 1st instar larvae) for *P. rivulare* and *Ps. frondale* (to assess comparability of *C. triangulifer* relative to other mayfly species). Each replicate had six treatments: control (Dyberry or White Clay Creek water) and five dilution concentrations (as % of produced-water). For 1st instar tests involving *C. triangulifer*, *P. rivulare* and *Ps. frondale*, produced-water concentrations were 0.0%, 0.195%, 0.391%, 0.781%, 1.563%, and 3.125%; for middle instar *C. triangulifer*, produced-water concentrations were 0.0%, 0.195%, 0.391%, 0.781%, 1.563%, 3.125%, and 6.250%. Change in conductivity was used to confirm dilution concentrations. Chemical composition of dilution water is presented in Figure 2 and Table 3; chemical composition of produced water and diluted produced water are presented in Figures 2 and 3. These were static (no renewal) experiments conducted at 20°C for 48 h. 20µL of diatom slurry was provided as food for each test vessel. Response assessed was survivorship (%, LC50). For comparability with acute RT tests, produced-water concentrations were also expressed as specific conductance (a common measure of dissolved ion concentrations) and mg Cl/L (chloride is a known, often regulated toxin in fresh water ecosystems).

Experiment 5: Chronic (whole lifecycle) toxicity test of produced-water

Experiment 5 was designed to quantify chronic responses of *C. triangulifer* in whole lifecycle exposures to produced water, and compare these results to data for two other mayfly species (i.e., *P. rivulare* and *Ps. frondale*), and for two standard test species (*C. dubia* and *P. promelas*). We conducted six chronic toxicity tests of produced-water (consisting of four replicates of 50 individuals each; Table 1) using either Dyberry or White Clay Creek water. These were whole-life tests that began with the introduction of newly hatched 1st instar into each 1.9-L jars filled with water (static, no renewal) and an air stone to maintain oxygen saturation. Larvae were fed algae grown on acrylic plates, replenished as needed. . There were four replicates for both water sources. Each replicate had six treatments: control and 5 dilution concentrations (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%, in Dyberry or White Clay Creek water). Chemical composition of dilution water is presented in Figure 2 and Table 3; chemical composition of produced water and diluted produced water are presented in Figures 2 and 3. These were static (no renewal) tests with 30-60 day exposures; the duration depended on the species and temperature (20°C). Tests were run at 20°C until all remaining larvae had emerged as adults. Emerging adults were trapped in cages over the jars and collected daily. Basic water chemistry data on the last day of the tests are presented in Appendix 2. Chronic response variables that could be measured were survivorship (%, LC50, NOEC, LOEC), development time (days), final body size (mg dry mass), growth rate (1st instar to adult, per day), and PGR.

Statistical Analysis

For acute tests, individual replicates with <90% survivorship in the control were omitted from the analysis. The Probit model (SAS Institute Inc., Cary, NC, version 9.3) was used to estimate LC50 from the remaining replicates which were then evaluated individually for Chi-square Goodness of Fit. If a replicate had a significant *p* value in the Chi-square analysis and the calculated LC50 was an outlier, that replicate was omitted from the final analysis. Since the replicates in our tests met the EPA requirement of a minimum of 20 individual animals per test treatment, the LC50 values reported here represent the mean of individual LC50 values across all acceptable replicates.

For the whole life (chronic) mayfly tests, expected survivorship in the controls has not been determined, thus no reps were rejected from the probit analysis on this basis and survivorship was normalized to the controls. NOEC and LOEC was determined by ANOVA with Tukey's post hoc tests (SAS Institute Inc., Cary, NC, version 9.3). IC25 was estimated using simple linear regression in the active portion of the dose response curve, where additional zeros beyond the lowest toxicant concentration resulting in zero survivorship were not included.

Results and Discussion

Water Chemistry

Water chemistry for the initial ambient and produced-water is shown in Tables 2 and 3 and Fig. 2). Other parameters (i.e., pH, temperature, conductivity, dissolved oxygen, ammonia, hardness and alkalinity) from samples taken at the termination of the whole life tests are presented in Appendices 1-3. At the end of Experiment 1 (Ambient water tests), ammonia levels appeared slightly elevated in a few treatments (e.g., Delaware River at Callicoon for *Ps. frondale*) although these levels were well below (about 2 orders of magnitude) levels reported as toxic to mayflies (Appendix 1). Water chemistry measured at the termination of Experiment 3 (chronic RT tests) and Experiment 5 (chronic produced-water tests) indicated no unusual values or notable differences between treatments for any of the parameters other than those resulting from the addition of the toxicant (Appendices 2 and 3).

Differences in ion concentrations among the ambient waters primarily reflect underlying geologies as well as land use/land covers. Ion composition of produced water was markedly different from that of ambient waters, both as absolute and relative concentrations (Tables 2 and 3; Figures 2 and 3). Compared to 29 other gas wells in the Marcellus Shale, produced-water from this study had high levels of total dissolved solids (TDS), chloride, barium, sodium, and strontium (Figure 4, Appendix 4).

Comparison of the RT NaCl and the produced-water treatments indicated that the relationship between conductivity and chloride differed only slightly among water sources and treatments (Figure 5). For a given chloride concentration, the produced-water had a somewhat higher conductivity than the RT water, no doubt due to the fact that several other ions in the produced-water treatments contributed significantly to the TDS (Figure 3). It is important to recognize that diluting the produced-water to a small percentage resulted in a relatively high conductivity, i.e., the produced-water was very concentrated. For example, a concentration of 1.56% produced-water resulted in a conductivity of >6000 µS/cm (Figure 6). Similarly, a concentration of only 0.25% produced water (see chronic produced-water experiments below) still resulted in an ion balance that was more similar to produced water than ambient water (Figures 2and 3).

Experiment 1: Whole-lifecycle (chronic) toxicity tests for ambient stream water

Survivorship

Survivorship during the chronic toxicity tests for ambient stream water ranged from 40-80% for *C. triangulifer* to 8-26% for *P. rivulare* and 2-36% for *Ps. frondale* (Figure 7). There was no significant difference in survivorship among the five ambient waters tested. However, survivorship for all three species appeared lower for the Dyberry and White Clay Creeks ambient-water treatments relative to similar treatments (i.e., Dyberry and White Clay Creek controls) conducted as part the chronic RT and chronic produced-water tests (see Consistency Analyses below).

Larval development time (1^{st} instar to adult, in days)

Development time during the chronic toxicity tests for ambient stream water ranged from 31-34 d for *C. triangulifer* to 28-33 d for *P. rivulare* and 29-42 d for *Ps. frondale* (Figure 8). There was no significant difference in development time among the five ambient waters tested. However, development time for all three species appeared longer by a few days for the Dyberry and White Clay Creeks ambient water treatments relative to similar treatments (i.e., Dyberry and White Clay controls) conducted as part the chronic RT and chronic produced-water tests (see Consistency Analyses below).

Median adult size (dry mass)

For all three species there were no significant difference in adult body size among the five ambient waters tested (Figure 9). Unlike survivorship and development time, body size in these ambient water tests were similar to RT and produced-water tests conducted later.

The absence of differences among ambient water treatments for survivorship, development time, and adult body size suggests that none of the waters tested contained toxins in sufficient quantities to affect these mayflies significantly. Lower survivorship and longer development time relative to controls of the chronic RT and produced-water tests suggest that conditions during the ambient water tests were somewhat different than in the controls in the later tests using produced-water. Because all of these tests were conducted in similar laboratory conditions our only explanation at this time is that food quality differed among the tests. The mayflies are fed a natural assemblage of algae that grows in a continuous supply of White Clay Creek water at ambient temperatures. It is possible that natural seasonality (e.g., March versus August) in this algal assemblage can affect survivorship and development time.

Experiment 2: Acute Reference Toxicant (RT) Tests with NaCl

The acute RT tests were run in three groups, where each of the test species, 1st and middle instar *C. triangulifer* and 1st instar *P. rivulare* and *Ps. frondale,* were run simultaneously. Results from the individual replicates were assessed separately, and replicates with <90% survivorship in the control (a total of 7 reps) were omitted from the analyses. In addition, eight replicates that had a significant Goodness-of-Fit result for the Probit model were examined to determine if the significant difference reflected a meaningful divergence from the expected Probit model shape. In all cases the estimated LC50s for those replicates were similar to the others, thus none were omitted from the analyses.

Survivorship (%)

Centroptilum triangulifer

Survivorship in the acute RT was assessed for both 1st and middle instar *C. triangulifer*, in both Dyberry and White Clay waters. Only one replicate (1st instar in Dyberry water) had survivorship <90% and was excluded from the analyses. 1st instar larvae of *C. triangulifer* were more sensitive than middle instar larvae when exposed to elevated NaCl concentrations, and both size classes were more sensitive to elevated salt concentrations in Dyberry relative to White Clay water (Table 4). The LC50 for 1st instar larvae was 2346 mg NaCl/L in Dyberry water versus 4054 mg NaCl/L in White Clay water; the LC50 values for middle instar larvae was 4771 mg NaCl/L in Dyberry water and 5527 mg NaCl/L in White Clay water. Our acute LC50 values for both 1st and middle instar *C. triangulifer* are markedly higher than was reported for 1st *C. triangulifer* in an unpublished RT conducted by EPA (659 mg NaCl/L; Struwing et al. in Table 4).

Procloeon rivulare

Survivorship in the acute RT was assessed for 1st instar *P. rivulare*, in both Dyberry and White Clay waters. Only one replicate (in Dyberry water) had survivorship <90% and was excluded from the analyses. Similar to *C. triangulifer*, *P. rivulare* was more sensitive to elevated NaCl concentrations in Dyberry water relative to White Clay water (Table 4). The LC50 for 1st instar larvae was 787 mg NaCl/L in Dyberry water versus 2476 mg NaCl/L in White Clay water. Both of these results suggest that 1st instar *P. rivulare* is more sensitive than 1st instar *C. triangulifer* in acute exposures to elevated NaCl concentrations (i.e., 787 versus 2346 mg NaCl/L in Dyberry water; 2476 versus 4054 mg NaCl/L in White Clay water). The difference between Dyberry and White Clay waters was evident for *C. triangulifer* and *P. rivulare* with greater NaCl tolerance in White Clay water than Dyberry (see below).

Pseudocloeon frondale

Survivorship in the acute RT was assessed for 1st instar *Ps. frondale*, in both Dyberry and White Clay waters. Five replicates (three in Dyberry water, two in White Clay water) had survivorship <90% and were excluded from the analyses. Similar to *C. triangulifer* and *P. rivulare*, *Ps. frondale* was more sensitive to elevated NaCl concentrations in Dyberry water relative to White Clay water (Table 4). For example, the LC50 for 1st instar *Ps. frondale* was 1626 mg NaCl/L in Dyberry water versus 3012 mg NaCl/L in White Clay water. The acute RT response for *Ps. frondale* was intermediate between the more sensitive P. *rivulare* and the more tolerant *C. triangulifer* (e.g., 1626 mg NaCl/L versus 787 mg NaCl/L for *P. rivulare* and 2346 mg NaCl/L for *C. triangulifer* in Dyberry water; 3012 mg NaCl/L versus 2476 for *P. rivulare* and 4054 mg NaCl/L for *C. triangulifer* in White Clay water).

Experiment 3: Chronic (whole lifecycle) Reference Toxicant (RT) Tests with NaCl

The chronic RT tests with NaCl were conducted with only 1st instar of *C. triangulifer*. *P. rivulare* and *Ps. frondale* were not examined in these experiments. Although LC50 is generally not determined in chronic tests, we included it because the dose response curve in our tests was appropriate for Probit analyses (once survivorship was normalized to the controls) and we felt it provided a useful comparison to the acute LC50s. We used simple linear regression (in the active portion of the dose response curve, where additional zeros associated with zero survivorship were not included after the first zero) to estimate IC25 (25% inhibition concentration) for development time, adult body weight, IGR, and PGR.

Survivorship (%)

The no-observed-effect concentration (NOEC) for survivorship of *C. triangulifer* was 412 mg NaCl/L in Dyberry water, and 824 mg NaCl/L in White Clay water (Figure 10). However, survivorship dropped to <10% in the next higher treatments. Thus, 824 and 1647 mg NaCl/L were the lowest-observed-effect concentration (LOEC) for *C. triangulifer* in Dyberry and White Clay waters, respectively.

The chronic LC50 for *C. triangulifer* exposed to elevated concentrations of NaCl was 651 mg NaCl/L in Dyberry water, and 1007 mg NaCl/L in White Clay water (Table 4). Both values were 25-28% of the acute LC50 values estimated for 1st instar *C. triangulifer* (2346 mg NaCl/L in Dyberry water, 4054 mg NaCl/L in White Clay water) and 14-18% of the acute LC50 values estimated for middle instar *C. triangulifer* (4771 mg NaCl/L in Dyberry water and 5527 mg NaCl/L in White Clay water). Our chronic LC50 value for *C. triangulifer* in WCC (1007 mg NaCl/L) is 21% higher than the chronic LC50 reported for *C. triangulifer* in moderately hard water in an unpublished RT conducted by EPA (833 mg NaCl/L; Struwing et al. in Table 4).

Development time (median days from 1st instar to adult emergence)

The NOEC for development time of *C. triangulifer* was a concentration of 206 mg NaCl/L in Dyberry and White Clay waters (Figures 11 and 12). However, development time increased significantly (1-1.5 d) in the next higher treatments. Thus, 412 mg NaCl/L was the LOEC for *C. triangulifer* in Dyberry and White Clay waters. We used simple linear regressions to estimate the IC25 for development time (i.e., the NaCl concentration needed to increase development time for *C. triangulifer* by 25%). The IC25 for development time was 565 mg NaCl/L for Dyberry water and 809 mg NaCl/L for White Clay water (Table 4). The highest concentration of NaCl (824 mg/L) increased development time 4 d compared to the control; development time ranged from ≈27 d for the control to 40 d in Dyberry water and 36 d in White Clay water (Figures 11 and 12).

Adult body size (mg dry mass)

The NOEC for adult body size of *C. triangulifer* was a concentration of 206 mg NaCl/L in Dyberry water (Figures 11 and 12). Thus, 412 mg NaCl/L was the LOEC for *C. triangulifer* in Dyberry, although the increase in body size would generally not be considered adverse. No differences in adult body size were observed among the NaCl treatments in White Clay waters, making the NOEC for adult body size of *C. triangulifer* a concentration of 824 mg NaCl/L in White Clay water (none survived at 1647 or 3295 mg NaCl/L). The IC25 for adult body size was 959 mg NaCl/L for Dyberry water and 799 mg NaCl/L for White Clay water (Table 4). In Dyberry Creek water, adult body size increased by 17% between the control and the highest NaCl concentration (824 mg/L): individuals in the control averaged 1.57 mg and the RT treatment averaged 1.89 mg. Adult biomass in White Clay averaged 1.72 mg across all treatments. Our IC25 value for adult body size of *C. triangulifer* in WCC (799 mg NaCl/L) is 249% higher than the IC25 reported for *C. triangulifer* in moderately hard water in an unpublished RT conducted by EPA (229 mg NaCl/L; Struwing et al. in Table 4).

Instantaneous growth rate (d^{-1})

The NOEC for IGR of *C. triangulifer* was a concentration of 206 mg NaCl/L in Dyberry and White Clay waters (Figures 11 and 12). Thus, 412 mg NaCl/L was the LOEC for *C. triangulifer* in Dyberry and White Clay waters. The IC25 for IGR was 654 mg NaCl/L for Dyberry water and 679 mg NaCl/L for White Clay water (Table 4).

Population Growth Rate (offspring individual^{-1} d^{-1})

C. *triangulifer* had a NOEC for PGR (progeny produced per individual per day) of 412 mg NaCl/L for Dyberry and White Clay waters (Figures 11 and 12). The LOEC was also the same for both waters at 824 mg NaCl/L. The LOEC represented a loss of approximately 44 progeny per individual per day (a loss of 91%) in Dyberry water and 19 (a loss of 42%) in White Clay water compared to the controls. The IC25 for PGR was estimated at 362 mg NaCl/L for Dyberry water and 517 mg NaCl/L for White Clay water (Table 4).

Experiment 4: Acute toxicity tests of produced-water

The acute produced-water tests were run in two groups, where each of the test species, 1st and middle instar *C. triangulifer* and 1st instar *P. rivulare* and *Ps. frondale*, were run simultaneously. Results from the individual replicates were assessed separately, and replicates with <90% survivorship in the control (a total of 8 reps) were omitted from the analyses. In addition, six replicates that had a significant Goodness-of-Fit result for the Probit model were examined to determine if the significant difference reflected a meaningful divergence from the expected Probit model shape. In one case the estimated LC50 for that replicate was different enough from the others that it was omitted from the analyses.

Survivorship (%)

Centroptilum triangulifer

No replicates using *C. triangulifer* had survivorship <90%, thus all four replicates (two tests with two replicates each) were averaged for each LC50 estimate. First and middle instar larvae of *C. triangulifer* had generally similar responses when exposed to elevated concentrations of produced-water, and both size classes generally responded similarly in Dyberry and White Clay water (Table 5). For example, the LC50 for 1st instar larvae was 1.764% produced-water in Dyberry water versus 1.988% produced-water in White Clay water. Similarly, the LC50 for middle instar larvae was 1.704% produced-water in Dyberry water and 1.496% produced-water in White Clay water.

Procloeon rivulare

Survivorship in the acute produced-water test was assessed for 1st instar *P. rivulare*, in both Dyberry and White Clay Creek waters. Two replicates using *P. rivulare* in Dyberry water were excluded from the analyses, one for low survivorship in the control and the other for poor fit to the Probit model. Thus, LC50 estimates for *P. rivulare* are the average of two replicates for the Dyberry waters. One replicate in the White Clay water was eliminated due to <90% survivorship in the control, thus the LC50 estimate for White Clay represents the average of three replicates. The LC50 for 1st instar *P. rivulare* larvae was 0.782% produced-water in Dyberry water and 0.735% produced-water in White Clay water (Table 5). These results suggest that 1st instar *P. rivulare* are more sensitive than 1st instar *C. triangulifer* in acute exposures of produced-water (i.e., 0.782% versus 1.764% in Dyberry water; 0.735% versus 1.988% in White Clay water).

Pseudocloeon frondale

Survivorship in the acute produced-water test was assessed for 1st instar *Ps. frondale*, in both Dyberry and White Clay Creek waters. Three replicates using *Ps. frondale* in White Clay water and two in Dyberry water were excluded from the analyses because of survivorship <90% in the controls. Thus, LC50 estimates *Ps. frondale* are the average of two replicates for Dyberry water, and one replicate for White Clay water, so there were not enough replicates in the test to compare water types (Table 5). Nevertheless it looks like *Ps. frondale* had the same response as the other mayfly species with similar tolerance to produced-water in White Clay water versus Dyberry water. The LC50 for 1st instar larvae of *Ps. frondale* was 0.251% produced-water in Dyberry water versus 0.272% produced-water in White Clay water. Both of these results suggest that 1st instar *Ps. frondale* is more sensitive than 1st instar *C. triangulifer* and *P. rivulare* in acute exposures to elevated concentrations of produced-water (e.g., 0.251% versus 1.764% and 0.782% in Dyberry water, respectively; 0.272% versus 1.988% and 0.735% in White Clay water, respectively).

Experiment 5: Chronic (whole lifecycle) toxicity test of produced-water

The chronic test with produced-water was conducted beginning with 1st instar larvae of *C. triangulifer*, *P. rivulare*, and *Ps. frondale*. IGR and development time for *P. rivulare* and *Ps. frondale* were calculated for males and females separately. Two test vessels failed and were eliminated from the analysis (jar 2007, control, Dyberry water, *C. triangulifer*; jar 1972, 0.391% produced-water, White Clay water, *C. triangulifer*). Analyses were conducted based on four replicates. As with the chronic RT (Experiment 3), Probit analyses of larval survivorship through a whole lifecycle (normalized to the controls) were used to estimate an LC50 while simple linear regression (in the active portion of the dose response curve; extra zeros were not included) was used to estimate IC25 for development time, adult body weight, IGR, and PGR.

Survivorship (%)

Centroptilum triangulifer

The NOEC for survivorship of *C. triangulifer* was 0.195% produced-water in Dyberry water and 0.391% produced-water in White Clay water (Figure 13). However, survivorship dropped to <60% in the next higher treatment for Dyberry water, and 0% survivorship for White Clay water. Thus, a produced-water concentration of 0.391% was the LOEC for *C. triangulifer* in Dyberry water and 0.781% for White Clay water.

The chronic LC50 for *C. triangulifer* was 0.373% produced-water in Dyberry water, and 0.415% in White Clay water (Table 5). Both values were 20-21% of the acute LC50 values estimated for 1st instar *C. triangulifer* (1.877% in Dyberry water, 2.005% in White Clay water) and 21-27% of the acute LC50 values estimated for middle instar *C. triangulifer* (1.813% in Dyberry water and 1.529% in White Clay water) (Table 5).

Procloeon rivulare

Determining the NOEC for survivorship of *P. rivulare* was difficult because survivorship initially increased with the addition of a small amount of produced-water, and then we observed a normal dose response curve (Figure 13). If we start at the peak of the survivorship curve (0.098%), the NOEC for survivorship of *P. rivulare* was 0.195% produced-water in Dyberry and White Clay waters. However, survivorship dropped to 35-45% in the next higher treatment. Thus, a produced-water concentration of 0.391% was the LOEC for *P. rivulare* in Dyberry and White Clay waters.

The chronic LC50 for *P. rivulare* was 0.372% produced-water in Dyberry Creek water, and 0.409% in White Clay Creek water (Table 5). These values were 49% and 59% of the acute LC50 values estimated for 1st instar *P. rivulare* (0.752% in Dyberry water, 1.060% in White Clay water). Chronic produced-water LC50 values for *P. rivulare* were similar to those estimated for *C. triangulifer* in Dyberry water (0.372% versus 0.373%), and in White Clay water (0.409% versus 0.415%).

Pseudocloeon frondale

The NOEC for survivorship of *Ps. frondale* was 0.195% produced-water in Dyberry and White Clay water (Figure 13). A produced-water concentration of 0.391% was the LOEC for *Ps. frondale* in Dyberry and White Clay water.

The chronic LC50 for *Ps. frondale* was 0.336% produced-water in Dyberry water, and 0.257% in White Clay water (Table 5). The chronic LC50 for produced-water estimated for 1st instar *Ps. frondale* in Dyberry water was somewhat greater than the acute LC50 value (0.336% versus 0.215%) while the chronic LC50 estimated for White Clay water was similar to the acute LC50 value (0.257% versus 0.268%). Chronic produced-water LC50 values for *P. rivulare* were similar to the chronic LC50 values estimated for *C. triangulifer* and *P. rivulare* in Dyberry Creek water (0.336% versus 0.372% and 0.373%), and lower than estimates for those species in White Clay Creek water (0.257% versus 0.409% and 0.415%).

Development time (median days from 1st instar to adult emergence)

Centroptilum triangulifer

The NOEC for development time of *C. triangulifer* was 0.098% produced-water in Dyberry water and 0.195% produced-water in White Clay water (Figure 14). Thus, a produced-water concentration of 0.195% was the LOEC for *C. triangulifer* in Dyberry water and 0.391% in White Clay water. The IC25 for development time of *C. triangulifer* was 0.199% produced-water in Dyberry water and 0.302% in White Clay water (Table 5). The highest concentration of produced water (0.391%) increased development time 12-14 d compared to the control: development time increased from ≈29 d for the control to 43 d in Dyberry water and 41 d in White Clay water with 0.391% produced water.

Procloeon rivulare

Males and females of *P. rivulare* exhibited similar response curves for development time. The NOEC for development time of *P. rivulare* was 0.195% produced-water in Dyberry and White Clay water (Figure 14). Thus, a produced-water concentration of 0.391% was the LOEC for *P. rivulare* in Dyberry and White Clay waters.

The IC25 for development time for females of *P. rivulare* was 0.633% produced-water in Dyberry water, and 0.548% in White Clay water (Table 5). The IC25 for development time for males of *P. rivulare* was 0.904% produced-water in Dyberry water, and 0.525% in White Clay water. These chronic IC25 values for development time of *P. rivulare* females were greater than the chronic IC25 values estimated for *C. triangulifer* in Dyberry water (0.633 versus 0.199%), and in White Clay water (0.548% versus 0.302%). Development time was increased by ≈5-6 d when the control was compared to the produced water (0.391%): development time increased from ≈28 d for the control to ≈33 d for both stream waters with 0.391% produced water.

Pseudocloeon frondale

Males and females of *Ps. frondale* exhibited similar response curves for development time. The NOEC for development time of *Ps. frondale* was 0.0% produced-water for females and 0.098% for males in Dyberry water (Figure 14). Conversely, the NOEC for development time of *Ps. frondale* was 0.098% produced-water for females and 0% for males in White Clay water. Thus, for females of *Ps. frondale*, a produced-water concentration of 0.098% was the LOEC in Dyberry water and 0.195% in White Clay water. For males of *Ps. frondale*, a produced-water concentration of 0.195% was the LOEC in Dyberry, and 0.098% in White Clay water. These LOEC values were lower than were observed for *C. triangulifer* and *P. rivulare*.

The IC25 for development time for females of *Ps. frondale* was 0.275% produced-water in Dyberry water and 0.337% in White Clay water (Table 5). The IC25 for development time for males of *P. rivulare* was 0.184% produced-water in Dyberry water and 0.282% in White Clay water. The chronic IC25 values for development time of *Ps. frondale* females were less than the chronic IC25 values estimated for female *P. rivulare* and greater than values for *C. triangulifer* in Dyberry water (0.275% versus 0.633 and 0.199%, respectively), and in White Clay water (0.337 versus 0.548% and 0.302%, respectively). Development time was increased on average by 9-12 d when the control was compared to the produced water (0.391%): development time increased from ≈29 d for the control to 38 d for females and 40 d for males in 0.391% produced water. Development time looked to be longer by 1-4 d in Dyberry water versus White Clay water when comparing similar sexes (Figure 14).

Adult body size (mg dry mass)

No differences in adult body size were observed for *C. triangulifer*, *P. rivulare*, and *Ps. frondale* in Dyberry water, and *C. triangulifer* in White Clay water (Figure 15). Differences among treatments were observed for both males and females of *P. rivulare* and *Ps. frondale* in White Clay water, but all differences were increases relative to the control. We interpreted these increases in body size not as adverse effects but as a response to lower densities surviving in higher concentrations of produced-water. As a result, we did not identify a NOEC or LOEC for adult body size reacting negatively to exposure to produced-water.

Instantaneous growth rate (d^{-1})

Centroptilum triangulifer

The NOEC for IGR of *C. triangulifer* was 0.098% produced-water in Dyberry water and 0.195% produced-water in White Clay water (Figure 16). Thus, a produced-water concentration of 0.195 % was the LOEC for *C. triangulifer* in Dyberry water and 0.391% for White Clay water.

The IC25 for IGR of *C. triangulifer* was 0.289% produced-water in Dyberry water, and 0.411% in White Clay water (Table 5). IGR for *C. triangulifer* decreased on average 31% between the control and the produced-water (0.391%); IGR was 0.26 d^{-1} for the control and 0.18 d^{-1} for the 0.391% produced water.

Procloeon rivulare

Males and females of *P. rivulare* exhibited similar response curves for IGR. The NOEC for *P. rivulare* was 0.195% produced-water in Dyberry and White Clay waters (Figure 16). Thus, a produced-water concentration of 0.391% was the LOEC for *P. rivulare* in Dyberry and White Clay waters.

The IC25 for IGR for females of *P. rivulare* was 0.711% produced-water in Dyberry water, and 0.690% in White Clay water (Table 5). The IC25 for development time for males of *P. rivulare* was 1.020 % produced-water in Dyberry water, and 0.678% in White Clay water. IGR for *P. rivulare* decreased between the control and the produced-water (0.391%) on average by 14%: IGR was 0.26 d^{-1} for the control and 0.22 d^{-1} for the 0.391% produced water. All of these IC25 values are greater than the maximum concentrations in which these larvae survived in our tests. Thus, these chronic IC25 values for growth rate of *P. rivulare* are not a sensitive enough indicator and IC10 maybe more informative for *P. rivulare*.

Pseudocloeon frondale

Males and females of *Ps. frondale* exhibited similar response curves for IGR. The NOEC for *Ps. frondale* was 0% produced-water for both males and females in Dyberry water, and for males in White Clay water (Figure 16). The NOEC for females in White Clay water was 0.098%. A produced-water concentration of 0.098% was the LOEC for males and females of *Ps. frondale* in Dyberry and males of *Ps. frondale* in White Clay water. The LOEC for females of *Ps. frondale* in White Clay water was 0.195%.

The IC25 for IGR for females of *Ps. frondale* was 0.370% produced-water in Dyberry water, and 0.491% in White Clay water (Table 5). The IC25 for IGR for males of *Ps. frondale* was 292% produced-water in Dyberry water, and 0.428% in White Clay water. All of these IC25 values approach or are greater than the LC50s for *Ps. frondale*. Thus, as with *P. rivulare*, smaller changes in growth rate of *Ps. frondale* may be of ecological significance, as most larvae will have died at the IC25 levels (see below). The IGR for *Ps. frondale* decreased between the control and the produced-water (0.391%) by 24% and 19% for the females in Dyberry and White Clay waters, respectively, and 32% and 22% for the males in Dyberry and White Clay waters respectively. On average IGR was 0.24 d^{-1} for the controls and 0.19 d^{-1} for the produced water.

Population Growth Rate (eggs individual^{-1} d^{-1})

The differences among produced-water treatments for PGR were very similar to those observed for survivorship (Figures 13 and 17). It is important to note that PGR is zero when chronic survivorship is zero, so there are no data illustrated at produced-water concentrations >0.391%. We used simple linear regressions to estimate the IC25 (i.e., the NaCl concentration needed to reduce PGR by 25%). PGR was the most sensitive of the four chronic responses evaluated for the three mayfly species.

Centroptilum triangulifer

The NOEC for PGR of *C. triangulifer* was 0.195% produced-water in Dyberry and White Clay waters, which translates to an average of 43 eggs individual^{-1} d^{-1} (Figure 17). The LOEC was 0.391% of produced-water for *C. triangulifer* in Dyberry and White Clay waters, which averaged 20 eggs individual^{-1} d^{-1} or a decrease 50% from the NOEC.

The IC25 for PGR of *C. triangulifer* was 0.247% produced-water in Dyberry water and 0.291% in White Clay water (Table 5).

Procloeon rivulare

Similar to survivorship, determining the NOEC for PGR of *P. rivulare* was difficult because PGR initially increased with the addition of a small amount of produced-water, and then we observed a normal dose-response curve (Figure 17). If we start at the peak of the survivorship curve (i.e., 0.098%), the NOEC for survivorship of *P. rivulare* was 0.195% produced-water in Dyberry and White Clay waters or a PGR of 13 eggs individual^{-1} d^{-1}. A produced-water concentration of 0.391% was the LOEC for *P. rivulare* in Dyberry and White Clay waters or a PGR of 5 eggs individual^{-1} d^{-1}. No larvae survived at the next highest concentration of produced-water.

The IC25 for PGR of *P. rivulare* was 0.185% produced-water in Dyberry water and 0.267% in White Clay water (Table 5). The IC25 growth rates were lower for *P. rivulare* than *C. triangulifer* in Dyberry water (0.185% versus 0.247%) and White Clay water (0.267% versus 0.291%).

Pseudocloeon frondale

The NOEC for PGR of *Ps. frondale* was 0.195% produced-water in Dyberry and White Clay waters, which translates to a PGR of 11 eggs individual^{-1} d^{-1} (Figure 17). Thus, a produced-water concentration of 0.391% was the LOEC for *Ps. frondale* in Dyberry and White Clay waters, which translates to a PGR of 1 egg individual^{-1} d^{-1}. No larvae survived at the next highest concentration of produced-water.

The IC25 for PGR of *Ps. frondale* was 0.136% produced-water in Dyberry water and 0.139% in White Clay water (Table 5). The growth rate for *Ps. frondale* was relatively similar to estimates for *P. rivulare* and lower than *C. triangulifer* in Dyberry water (0.136% versus 0.185% and 0.247%, respectively), and lower than both other mayflies in White Clay water (0.139% versus 0.267% and 0.291%).

Consistency Analyses for Mayfly Performance in Controls Across Chronic Experiments

We have worked with *C. triangulifer* in laboratory rearings and have observed reasonably consistent survivorship, development times, and adult body size in most efforts. For Experiment 1, we noticed that survivorship was somewhat low and development time was a little long relative to our historical, somewhat anecdotal, observations. Although we had less experience working with them, we were concerned that the same was true for *P. rivulare* and *Ps. frondale*. To assess if there were differences in these basic life history characteristics among controls (treatments with no toxicant added) in the different chronic experiments (ambient and produced-water for all three species, RT for *C. triangulifer* only), we used a two-way ANOVA (experiment x dilution water) for each response measure [i.e., survivorship, adult body size, development time, instantaneous growth rate (IGR) and population growth rate (PGR)], for all three mayfly species. Tukey post-hoc tests were used for pairwise comparisons to identify individual differences among experiments and between dilution waters (Dyberry and White Clay Creek).

Survivorship (%)

No source water effect was observed for survivorship of the three mayfly species; however, there was an experiment effect for all three species. For *P. rivulare* and *Ps. frondale*, survivorship in the ambient experiment was lower than in the produced-water experiment (there were no RT experiment for these species). Although the ANOVA's experiment effect was significant for *C. triangulifer*, the Tukey test did not identify any significant pairwise differences.

Development time (median days from 1st instar to adult emergence)

No source water effect was observed for survivorship of the three mayfly species, and no experiment effect was observed for *P. rivulare* and females of *Ps. frondale*. For *C. triangulifer*, development time was longer in the ambient test than either the RT or produced-water experiments, but no differences were seen between RT or produced-water experiments. For male *Ps. frondale*, development time was longer in the ambient test than the produced-water experiment.

Adult body size (mg dry mass)

Both experiment and source water effects were observed for *C. triangulifer*, but none of the pairwise comparisons were significant. No experiment effect was observed for *Ps. frondale*, but both sexes exhibited a source water effect. *Ps. frondale* reared in Dyberry water were larger than those reared in White Clay water. No experiment or source water effects were observed for males or females of *P. rivulare*.

Instantaneous growth rate (d^{-1})

No source water effect was observed for instantaneous growth rate of the three mayfly species. Growth rates for *C. triangulifer* were higher in both the RT and produced-water experiments relative to the ambient waters experiment. Growth rates for male *Ps. frondale* were also higher in the produced-water experiments relative to the ambient waters experiment (no RT was performed for *Ps. frondale*). No experiment effects were observed for males or females of *P. rivulare* and female *Ps. frondale*.

Population Growth Rate (eggs individual^{-1} d^{-1})

No source water effect was observed for instantaneous growth rate of the three mayfly species. Population growth rates for *C. triangulifer* were higher in both the RT and produced-water experiments than in the ambient waters experiment. There were no differences between the ambient and produced-water experiments for population growth rate of *P. rivulare* or *Ps. frondale*.

Summary of 5 Experiments and Comparison of Mayflies to Other Test Species

Ambient Tests

The ambient results indicated that water quality among the five stream waters tested did not affect any of the three mayfly species – there were no significant differences among ambient water treatments for survivorship, development time or adult size. In the ambient tests, chronic survivorship for *P. rivulare* and *Ps. frondale* was lower, development time for *C. triangulifer* and male *Ps. frondale* was longer, instantaneous growth rates for *C. triangulifer* and male *Ps. frondale* were slower, and population growth rates for *C. triangulifer* were lower relative to the other experiment(s). A natural assemblage of algae grown in White Clay Creek water in our greenhouse is used to feed mayflies in all our tests. The low survivorship, longer development times, slower instantaneous, and population growth rates in the ambient water tests may have been due to differences in food quality available at the time of year this test was done (late March and early April vs. mid-Summer for the others).

Reference Toxicant (RT) Tests

Acute tests for the reference toxicant NaCl were performed for all three mayfly species in both Dyberry Creek and White Clay Creek waters (Figures 18 and 19). NaCl toxicity clearly differed among the three species tested, and it depended on the dilution waters used. *C. triangulifer* was the least sensitive of the three mayflies, and middle instar *C. triangulifer* were less sensitive than 1st instars (Table 4, Figure 18). NaCl was consistently more toxic (i.e., lower LC50) in Dyberry water than in White Clay water (Table 4, Figure 18). Chronic (entire life cycle) tests were performed only for *C. triangulifer*. As expected, the chronic LC50s for the *C. triangulifer* RT test were much lower than the acute values. In Dyberry water the chronic LC50s were about 28% of the acute values for first instars (651 vs. 2346 mg NaCl/L) and only 14% of the values for middle instars (4771 mg/L). In White Clay water the chronic LC50s were 25% and 18% of acute values for first and middle instars (1007 mg/L for chronic, 4054 for first instar acute and 5527 for middle instar acute). Our acute tests ran 48 h, at the end of which some of the mayfly individuals in treatments that had only partial mortality appeared to be dying, but because they still showed movement, were scored as alive (this is the same interpretative approach as was used for *Ceriodaphnia dubia*. Had our acute tests run another day (72 h; cf Canedo-Arguelles et al. 2012), the resulting LC50s would likely have been lower, reducing the difference between the acute and chronic LC50s.

The fathead minnow *Pimephales promelas* and the water flea *Ceriodaphnia dubia* are commonly used to assess toxicity and set effluent limits. In acute tests using the same dilution waters we used for our mayflies, *P. promelas* had much higher LC50s (7787 mg NaCl/L in Dyberry water and 7071 mg NaCl/L in White Clay water) than the other taxa, which ranged from 787 mg NaCl/L for *P. rivulare* in Dyberry water to 5527 mg NaCl/L for *C. triangulifer* (middle instar) in White Clay water (Table 4, Figure 18). LC50s for *P. promelas* were ≈2-9 times higher than those for mayflies. *C. dubia* had LC50s that were higher than all 1st instar mayflies in Dyberry water (2672 mg NaCl/L versus 2346, 787, and 1626 mg NaCl/L for *C. triangulifer, P. rivulare,* and *Ps. frondale*, respectively) but only higher than *P. rivulare* in White Clay water (2736 versus 4054, 2476, 3012 mg NaCl/L for *C. triangulifer, P. rivulare,* and *Ps. frondale*, respectively). LC50s for *C. dubia* in both White Clay and Dyberry water were comparable to those previously observed for moderately hard waters (Table 4).

Chronic tests with Dyberry and White Clay dilution waters were not performed for *P. promelas* and *C. dubia*. However, EPA ECOTOX values for *P. promelas* and *C. dubia* in chronic SRT tests with NaCl were available for comparison with *C. triangulifer* (R. MacGillivray, DRBC, personal communication).

NOEC values for these taxa were reported as 1378 mg NaCl/L for *P. promelas* and 644 mg NaCl/L for *C. dubia*, which were both higher than the NOEC value of 206 mg NaCl/L and the LOEC value of 412 mg NaCl/L we report for *C. triangulifer* (Table 4). IC25 was also lower for *C. triangulifer* than for *P. promelas* and for *C. dubia*: 1422 mg NaCl/L for *P. promelas* and 908 mg NaCl/L for *C. dubia* compared with 362 and 517 mg NaCl/L for *C. triangulifer* (based on population growth rate) in Dyberry and White Clay waters, respectively.

In all of our acute and chronic mayfly tests, LC50s were lower for Dyberry water than for White Clay water (Table 4, Figure 18). This may reflect differences in hardness between Dyberry (soft) and White Clay Creek (moderately hard) dilution water (Figure 2). Chloride toxicity has been found to decrease with increasing hardness (e.g., Cowgill and Milazzo 1990, Mount et al. 1997, Soucek 2007, Soucek et al. 2011). This response to water source (hardness) was not evident in the acute tests with *C. dubia* and *P. promelas* conducted at the same time as the mayfly experiments (Table 4, Figure 18), which highlights that *C. dubia* did not respond to the differences in hardness between the two creek waters as did the mayflies, and as has been published (e.g., Mount et al. 1997, Soucek 2007, Soucek et al. 2011).

Produced-water Tests

LC50s in the acute study indicated *C. triangulifer* (1^{st} and middle instars) was more tolerant of produced-water than the other two mayflies (Figure 18, Tables 4 and 5). In the acute test, *C. triangulifer* (1^{st} and middle instars) had LC50s that ranged from 1.529% to 2.005% produced-water while LC50s for *P. rivulare* and *Ps. frondale* ranged from 0.215% to 1.060% produced-water. The response of *P. promelas* and *C. dubia* for the acute produced-water test was similar to *P. rivulare*. LC50s for *P. promelas* and *C. dubia* were 0.630% and 0.590% in Dyberry water, and 0.970% and 1.000% in White Clay water, respectively. Of the five species examined, *Ps. frondale* appeared to be the most sensitive to produced-water with a LC50 of 0.215% in Dyberry water and 0.268% in White Clay water. In general, acute LC50s were higher in White Clay water versus Dyberry water with the exception of middle instar of *C. triangulifer*, which had the opposite response. However, the LC50 differences between dilution waters was less than was observed for the NaCl RT (Figure 18), presumably because the addition of the produced water, even as a fraction of 1%, adds enough anions and cations that the two treatment waters that are now more similar to each other (i.e., as dilute produced water) than were the original source waters (Figures 2 and 3). As with the NaCl RT, we suspect that acute LC50 numbers using produced-water would have been lower for the mayfly species if the study had been conducted for 72 h instead of 48 h because we observed many individuals were near death (but counted as viable) when the experiment was terminated at 48 h. For future tests we recommend a 72 hour duration.

Comparison of the acute and chronic studies with produced-water indicated that LC50s for the chronic study were much lower than the acute study for *C. triangulifer* and *P. rivulare*, but not *Ps. frondale* (Figure 18, Table 5). However, LC50s from the acute and chronic tests for *Ps. frondale* indicated produced-water concentrations of 0.215% vs. 0.336% in Dyberry water and 0.268% vs. 0.257% in White Clay water, respectively. Chronic values for *C. triangulifer* and *P. rivulare* were slightly higher in White Clay water than Dyberry water but the opposite was true for *Ps. frondale*. Comparison of chronic LC50s for the *C. triangulifer* and *P. rivulare* were 0.373% and 0.372% in Dyberry water, and 0.415% and 0.409% in White Clay water, respectively. IC50s for chronic survivorship with produced-water 0.059% and 0.115% for *P. promelas* in Dyberry and White Clay water, respectivey. These values appear to be significantly lower than the mayflies. In contrast, comparable IC50s for survivorship in *C. dubia* were 1.444% and 1.167%, considerably higher than the mayflies.

For the mayflies, IC25 (based on PGR) for chronic produced-water indicated *C. triangulifer* had higher tolerance of produced-water than *P. rivulare* and *Ps. frondale* (Figure 19). IC25 for females of *C. triangulifer, P. rivulare*, and *Ps. frondale* were 0.247%, 0.185% and 0.136% in Dyberry water, respectively, and 0.291%, 0.267% and 0.139% in White Clay water, respectively (Table 5). IC25 (based on growth) for *P. promelas* indicated this species was more sensitive to produced-water than the three mayfly species: IC25 for *P. promelas* was 0.040% in Dyberry water and 0.080% in White Clay water. In contrast, IC25 (based on reproduction) for *C. dubia* (0.500% in Dyberry water and 0.550% in White Clay water) was higher than the IC25 for any of the other four species – the IC25 for *C. dubia* was actually higher than the chronic LC50s for all three mayflies. IC25s were higher in White Clay water than Dyberry water for all the species, with the possible exception of *Ps. frondale* that had only a minor difference between the stream waters.

Reference Toxicant versus Produced-Water Tests for Mayflies

Across the acute and chronic experiments, the results for the RT-NaCl may provide important insight into the results of the produced-water tests because chloride is the toxicant in the RT and the predominant anion in produced water. Dilution factor alone is unlikely to be an effective way of predicting effects on aquatic organisms because produced-waters vary considerably in composition (Figure 4). Specific conductivity and chloride are two other measures that could be good indicators of potential toxicity across different produced-water sources, especially if the addition of produced water dramatically changes the chemical composition of the receiving water. A comparison of point estimation measures of acute and chronic responses from the RT-NaCl and produced water tests (expressed as specific conductivity and Cl concentration; Figure 20) highlights two details: (1) dilution water affects toxicity far more in the RT test (along the x-axis) than in the produced-water test (y-axis), to the point that dilution water was only important for Instantaneous Growth Rate in the produced-water tests for *C. triangulifer*, and (2) response values expressed as conductivity or chloride concentration were similar between the RT-NaCl and produced-water tests for some species/dilution water treatments, but not for others. As was noted above, the addition of even a fraction of 1% produced water adds enough anions and cations that the different stream waters will be more similar to each other (i.e., as dilute produced water) than were the original source waters (Figures 2 and 3). We will need additional chronic data to understand whether the results for Instantaneous Growth Rate are consistent across species, streams, and produced waters. For chronic responses of *C. triangulifer*, differences between the RT-NaCl and produced-water tests were generally similar whether expressed as Cl or conductivity. *Centroptilum triangulifer* may be slightly more tolerant of elevated conductivity or Cl when it resulted from a produced-water rather than a NaCl addition. Conversely, *Ps. frondale* (and possibly middle instar *C. triangulifer*) appears less tolerant to acute exposures to elevated conductivity and Cl from a produced-water addition.

Choice of Mayfly Test Species

The acute experiments found that *P. rivulare* and *Ps. frondale* were more sensitive to elevated ion concentrations than *C. triangulifer* – this difference also apparent for population growth rate (but not some of the other variables measures) in the chronic tests. In addition, all three mayfly were as or more sensitive to elevated ion concentrations than the standard lab invertebrate *C. dubia*. While *C. triangulifer* maybe not the most sensitive mayfly examined, it has some advantages relative to other mayfly species. The life history, taxonomy and genetics of *C. triangulifer* are relatively well studied (Sweeney and Vannote 1984, Funk et al. 2006) and it has proven to be well adapted for monitoring and toxicity studies conducted in laboratory settings (Sweeney et al. 1993, J. Lazorchak, personal communication). In addition, chronic response variables had values that were less than or approximately equal to the chronic LC50 (Table 5). *Centroptilum triangulifer* is also a clonal parthenogen so sexual differences need not be accounted for in measurements (e.g., biomass, development time, growth, etc.) and maintenance of laboratory colonies is easier (e.g., mating is not necessary, offspring are identical, and inbreeding of lab populations is not an issue). Thus, *C. triangulifer* has the potential to provide important insights into ion toxicity, although water quality standards that are protective of *C. triangulifer* may not be protective of other mayfly species.

Response Variables for Mayfly Chronic Tests

We report the results of several variables (survivorship, development time, adult body size, individual growth rate, and population growth rate) because there are no EPA standards for chronic toxicity endpoints specific to *C. triangulifer, P. rivulare* or *Ps. frondale*. The most sensitive measure across the three species was population growth rate (PGR) (Tables 4 and 5; Figure 19). Other variables produced inconsistent or unusable results because they did not respond adequately (i.e., the IC25 was similar to or greater than the chronic LC50 – significant mortality occurred before the variable changed 25%). For example, for *C. triangulifer*, the lowest IC25 in produced water was for development time, and this was well below the chronic LC50 in produced water (Table 5). However, the IC25 for development time and instantaneous growth rate in produced water was greater than the chronic LC50 for *P. rivulare*. The IC25 for instantaneous growth rate in produced water was also similar to the chronic LC50 for *Ps. frondale*. Population growth rate combines overall performance (fecundity and development time) with survivorship; thus, an IC25 for PGR produced values that were consistently less than the chronic LC50.

Conclusions

All three species of mayflies tested are native to streams and rivers in the Marcellus region of Pennsylvania and were sensitive to produced water. Of the three, we believe *Centroptilum triangulifer* is the most suitable for this type of toxicity study for several reasons (see **Choice of Mayfly Test Species** above). However, in future we recommend lengthening the duration of acute tests from 48 to 72 hours. Relatively soft water such as that from Dyberry Creek is an appropriate dilution water for these tests because it is representative of surface waters in the region of concern (Delaware River Basin) and in chronic tests mayflies were slightly more sensitive to produced water when diluted with Dyberry Creek water (compared with the moderately hard water of White Clay Creek). In chronic tests, mayflies were more sensitive than daphniids (*C. dubia*), but fathead minnows were significantly more sensitive than either the mayflies or daphniids. The high sensitivity of fathead minnows in chronic tests was unexpected given their relatively high tolerance to chloride (the major constituent of produced waters). We recommend additional testing of produced water samples to further investigate variability in responses and the apparent sensitivity of fathead minnows to produced water.

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Table 1. Experimental design for 5 sets of toxicity tests with mayflies.

Test Animals †	Source Waters *	No. Dilutions	No. Tests	No. Reps	*n* Value for Analysis	No. Animals/ Vessel	Test Duration
Experiment 1: Ambient Waters (chronic)							
C.t. 1st instar	5	N/A	1	4	4	50	whole life
P.r. 1st instar	5	N/A	1	4	4	50	whole life
Ps.f. 1st instar	5	N/A	1	4	4	50	whole life
Measurements: survival, development time, dry mass, fecundity							
Endpoints: LOEC, NOEC, IC25, LC50							
Experiment 2: Reference Toxicant (NaCl; acute)							
C.t. 1st instar	Dyberry	5+ctrl	3	2	5	20	48h
	WCC	5+ctrl	3	2	6	20	48h
C.t. middle instar	Dyberry	5+ctrl	3	2	6	20	48h
	WCC	5+ctrl	3	2	6	20	48h
P.r. 1st instar	Dyberry	5+ctrl	3	2	5	20	48h
	WCC	5+ctrl	3	2	6	20	48h
Ps.f. 1st instar	Dyberry	5+ctrl	3	2	3	20	48h
	WCC	5+ctrl	3	2	4	20	48h
Measurements: survival							
Endpoints: LC50							
Experiment 3: Reference Toxicant (NaCl; chronic)							
C.t. 1st instar	Dyberry	5+ctrl	1	4	4	50	whole life
	WCC	5+ctrl	1	4	4	50	whole life
Measurements: survival, development time, dry mass, fecundity							
Endpoints: LOEC, NOEC, IC25, LC50							
Experiment 4: Produced Water (acute)							
C.t. 1st instar	Dyberry	5+ctrl	2	2	4	20	48h
	WCC	5+ctrl	2	2	4	20	48h
C.t. middle instar	Dyberry	5+ctrl	2	2	4	20	48h
	WCC	5+ctrl	2	2	4	20	48h
P.r. 1st instar	Dyberry	5+ctrl	2	2	2	20	48h
	WCC	5+ctrl	2	2	3	20	48h
Ps.f. 1st instar	Dyberry	5+ctrl	2	2	2	20	48h
	WCC	5+ctrl	2	2	1	20	48h
Measurements: survival							
Endpoints: LC50							
Experiment 5: Produced Water (chronic)							
C.t. 1st instar	Dyberry	5+ctrl	1	4	4	50	whole life
	WCC	5+ctrl	1	4	4	50	whole life
P.r. 1st instar	Dyberry	5+ctrl	1	4	4	50	whole life
	WCC	5+ctrl	1	4	4	50	whole life
Ps.f. 1st instar	Dyberry	5+ctrl	1	4	4	50	whole life
	WCC	5+ctrl	1	4	4	50	whole life
Measurements: survival, development time, dry mass, fecundity							
Endpoints: LOEC, NOEC, IC25, LC50							

† *C.t.* = *Centroptilum triangulifer*; *P.r.* = *Procloeon rivulare*; *Ps.f.* = *Pseudocloeon frondale*

* source waters for Exp. 1 were five ambient waters (see Table 1). For Experiments 2–5 dilution waters were Dyberry Creek (soft) and White Clay Creek (WCC; moderately hard)

Table 2. Water parameters from chronic study on five streams using ambient water. Values are initial water conditions at the beginning of the study (19 Mar 2012). '<DL' indicates value was below the method detection limit.

Parameter	Dyberry	White Clay	Delaware nr. Callicoon	West Branch Lackawaxen	Lackawaxen nr. Honesdale
Temperature (C) [a]	9.5	8.5	10.5	8.42	8.74
Dissolved Oxygen (ppm) [a]	12.88	na	13.99	13.68	13.84
pH [a]	6.26	7.52	7.39	6.30	6.16
Specific Conductance (µS/cm) [a]	49	235	56	62	81
Ammonia as N (mg/L)	0.025	0.026	0.015	0.234	0.155
Arsenic (µg/L)	0.13	<DL	0.20	0.21	0.24
Barium (µg/L)	20.6	52.5	21.8	17.1	26.1
Calcium (mg/L)	7.65	26.00	5.69	8.43	9.52
Chloride (mg/L)	2.75	10.90	6.58	4.98	8.94
Hardness, Total (mg/L)	22	105	19	26	28
Iron (µg/L)	72	124	69	93	126
Lithium (µg/L)	0.59	1.15	0.53	0.58	3.93
Magnesium (mg/L)	0.68	9.76	1.17	1.08	1.10
Manganese (µg/L)	12.5	14.1	16.4	13.6	43.2
Methylene Blue Active Substances (mg/L)	0.069	0.079	0.085	0.091	0.096
Nitrate + Nitrite as N (mg/L)	0.335	3.880	0.427	0.509	0.378
Nitrite as N (mg/L)	0.0084	0.0148	0.0095	0.0093	0.0106
Potassium (mg/L)	0.41	1.83	0.56	0.74	0.96
Sodium (mg/L)	2.34	7.25	4.78	3.77	6.62
Strontium (µg/L)	16	103	20	27	36
Sulfate (mg/L)	4.6	17.3	4.6	5.1	5.9
Total Alkalinity (mg/L)	50	55	10	25	8
Total Dissolved Solids (mg/L)	40	148	44	45	57
Total Kjeldahl Nitrogen (mg/L)	0.13	0.18	0.13	0.14	0.40
Total Suspended Solids (mg/L)	2	2	2	3	2
Turbidity (NTU)	1.05	1.10	1.65	1.68	1.79
Gross Alpha 1st count (pCi/L)	0.118	0.283	0.096	0.094	0.125
Gross Beta (pCi/L)	0.49	1.41	0.43	0.60	1.07

[a] Field measurements

Table 3. Water parameters for undiluted produced-water sampled 16 July 2012. '<DL' indicates value was below the method detection limit.

Parameter	
1,1-Bicyclohexyl (µg/L)	16
1-Propene, 2-fluoro- (µg/L)	20.5
3-Amino-4-methoxybenzamide (µg/L)	19.6
4-Propionyloxytridecane (µg/L)	18
Ammonia as N (mg/L)	160
Arsenic (µg/L)	<DL
Barium (µg/L)	12,900,000
Bromide (mg/L)	756
Calcium (mg/L)	18,800
Chemical Oxygen Demand (mg/L)	3400
Chloride (mg/L)	121,000
Chloroform (µg/L)	<DL
Chloromethane (µg/L)	1.07
Cyclohexanone, 2-methyl-5-(1-methylethenyl)- (µg/L)	17.2
Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)- (µg/L)	18.4
Cyclopentane, 1-ethyl-3-methyl- (µg/L)	49.2
DODECANE (µg/L)	77.8
Decane, 2,5,6-trimethyl- (µg/L)	32.6
Dimethyl Ether (µg/L)	31.3
Dodecane, 2-methyl- (µg/L)	23.1
Dodecane, 3-methyl- (µg/L)	26.8
Dodecane, 6-methyl- (µg/L)	48.5
Formamide (µg/L)	57.4
Hardness, Total (mg/L)	54,200
Heptylcyclohexane (µg/L)	25.3
Iron (µg/L)	50,600
Lithium (µg/L)	214,000
Magnesium (mg/L)	1750
Manganese (µg/L)	6880
Methylene Blue Active Substances (mg/L)	1.11
Methylene Chloride (µg/L)	<DL
Nitrate + Nitrite as N (mg/L)	1.99
Nitrite as N (mg/L)	0.793
Octane, 2,6-dimethyl- (µg/L)	43.2
Potassium (mg/L)	351
Sodium (mg/L)	59,200
Strontium (µg/L)	6,150,000
Sulfate (mg/L)	21.6
Tetradecane (01) (µg/L)	93.8
Tetradecane (02) (µg/L)	18.9
Total Alkalinity (mg/L)	55
Total Dissolved Solids (mg/L)	314,000
Total Kjeldahl Nitrogen (mg/L)	191
Undecane (µg/L)	17.7
Undecane, 3-methyl- (µg/L)	29.4
m,p-Xylene (µg/L)	1.55
o-Xylene (µg/L)	0.592
tert-Butyl alcohol (µg/L)	121
Gross Alpha (pCi/L)	19,000
Gross Alpha 1st count (pCi/L)	45,000
Gross Beta (pCi/L)	6000

Table 4. Reference Toxicant tests with NaCl done for acute (48 h) and chronic conditions using water from Dyberry and White Clay (WC) Creeks. Results for *P. promelas*, *C. daphnia* and *P. subcapitata* provided by Delaware River Basin Commission for Dyberry and WC and mayfly data provided by Stroud Water Research Center. 'Other' refers to results from other studies (see footnotes). Conductivity values are based on NaCl concentrations.

Parameter	Species	Life stage	NaCl (mg/L)			Cond. (µS/cm)	
			Other	Dyberry	WC	Dyberry	WC
ACUTE							
LC50	*Centroptilum triangulifer*	1st instar	659[a]	2346	4054	4406	7737
		middle instar		4771	5527	8829	10456
	Procloeon rivulare	1st instar	na	787	2476	1564	4825
	Pseudocloeon frondale	1st instar	na	1626	3012	3094	5811
	Pimephales promelas	8-10d old*	7050-8700[d]	7787*	7071*	14331*	13303*
	Ceriodaphnia dubia	<24h old	2504[a]	2672	2736	5001	5305
CHRONIC							
LC50	*Centroptilum triangulifer*		833[a]	651	1007	1313	2113
NOEC	*Centroptilum triangulifer* [b]			206	206	468	657
	Pimephales promelas		1378[c]				
	Ceriodaphnia dubia		644[c]/1571[a]				
LOEC	*Centroptilum triangulifer* [b]			412	412	901	1062
IC25	*Centroptilum triangulifer* [e]			362	517	789	1211
	Pimephales promelas		1422[c]				
	Ceriodaphnia dubia		908[c]				
IC25 development time	*Centroptilum triangulifer*			565	808	1158	1747
IC25 instantaneous growth	*Centroptilum triangulifer*			654	679	1321	1508
IC25 mean individual dry mass	*Centroptilum triangulifer*		229[a]	959	799	1876	1730

[a] Struwing et al. In preparation. Moderate hard water (80-100 mg/L hardness), 25C, 48h for acute tests.

[b] based on development time or instantaneous growth rate

[c] EPA ECOTOX (R. MacGillivray, pers. comm.)

[d] EPA ECOTOX (Ref #2145 Adelman and Smith 1976; 11 wks old fish, 48h)

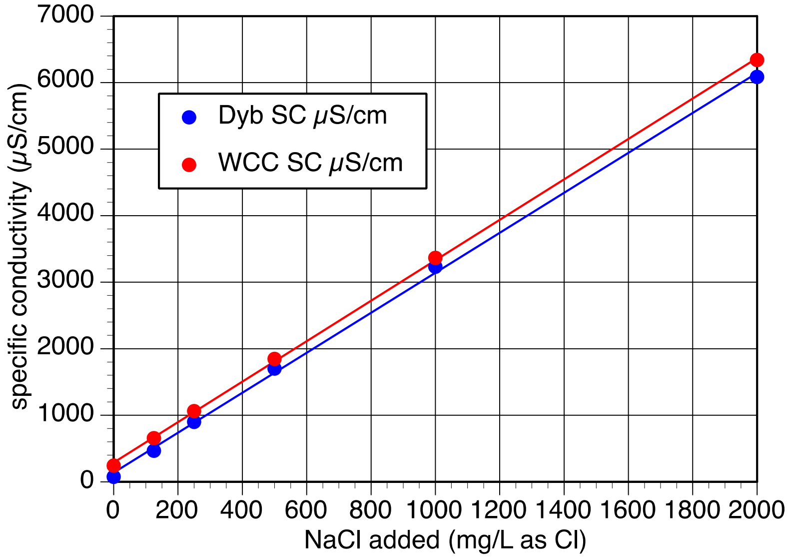
[e] based on population growth rate

Table 5. Produced-water results for acute (48 h) and chronic conditions done by Delaware River Basin Commission (*P. promelas*, *C. daphnia* and *P. subcapitata*) and Stroud Water Research Center (all mayflies). Tests conducted using water from Dyberry and White Clay (WC) Creeks. Conductivity values are based on NaCl concentrations.

Treatment	Taxon	Life stage or Sex	% Produced Water		Cl (mg/L)		Cond. (µS/cm)	
			Dyberry	WC	Dyberry	WC	Dyberry	WC
ACUTE								
LC50	*Centroptilum triangulifer*	1st instar	1.764	1.988	2150	2432	6992	7972
		middle instar	1.704	1.496	2077	1828	6757	6058
	Procloeon rivulare	1st instar	0.782	0.735	943	894	3144	3099
	Pseudocloeon frondale	1st instar	0.251	0.272	290	325	1063	1298
	Pimephales promelas	8-10d old	0.630	0.970	756	1182	2549	4012
	Ceriodaphnia dubia	<24h old	0.590	1.000	707	1219	2392	4129
CHRONIC								
LC50	*Centroptilum triangulifer*		0.373	0.415	440	501	1542	1854
	Procloeon rivulare		0.372	0.409	439	494	1538	1831
	Pseudocloeon frondale		0.336	0.257	395	307	1397	1240
IC50 survival	*Ceriodaphnia dubia*		1.444	1.167	1757	1424	5738	4779
	Pimephales promelas		0.059	0.115	54	133	311	687
IC25 development time	*Centroptilum triangulifer*	F	0.199	0.302	226	362	860	1415
	Procloeon rivulare	F	0.633	0.548	760	664	2560	2371
		M	0.904	0.525	1093	636	3622	2282
	Pseudocloeon frondale	F	0.275	0.337	320	405	1158	1551
		M	0.184	0.282	208	338	801	1337
IC25 growth	*Centroptilum triangulifer*[a]	F	0.289	0.411	337	496	1212	1838
	Procloeon rivulare[a]	F	0.711	0.690	856	839	2886	2923
		M	1.020	0.678	1236	824	4077	2877
	Pseudocloeon frondale[a]	F	0.370	0.491	436	594	1530	2150
		M	0.292	0.428	341	517	1224	1905
	Pimephales promelas		0.040	0.080	31	90	237	551
	Pseudokirchneriella subcapitata		0.080	0.060	80	65	393	473
IC25 PGR	*Centroptilum triangulifer*	F	0.247	0.291	285	349	1048	1372
	Procloeon rivulare	F	0.185	0.267	209	319	805	1278
	Pseudocloeon frondale	F	0.136	0.139	149	162	613	781
IC25 reproduction	*Ceriodaphnia dubia*		0.500	0.550	596	667	2039	2379

[a] measured as instantaneous growth

Means (n = 4 jars) of measured conductivity at start of chronic NaCl experiment with *C. triangulifer*



Dyberry f(x) = 3.004756E+0*x + 1.354286E+2
R^2 = 9.991446E-1

WCC f(x) = 3.040074E+0*x + 2.882857E+2
R^2 = 9.997646E-1

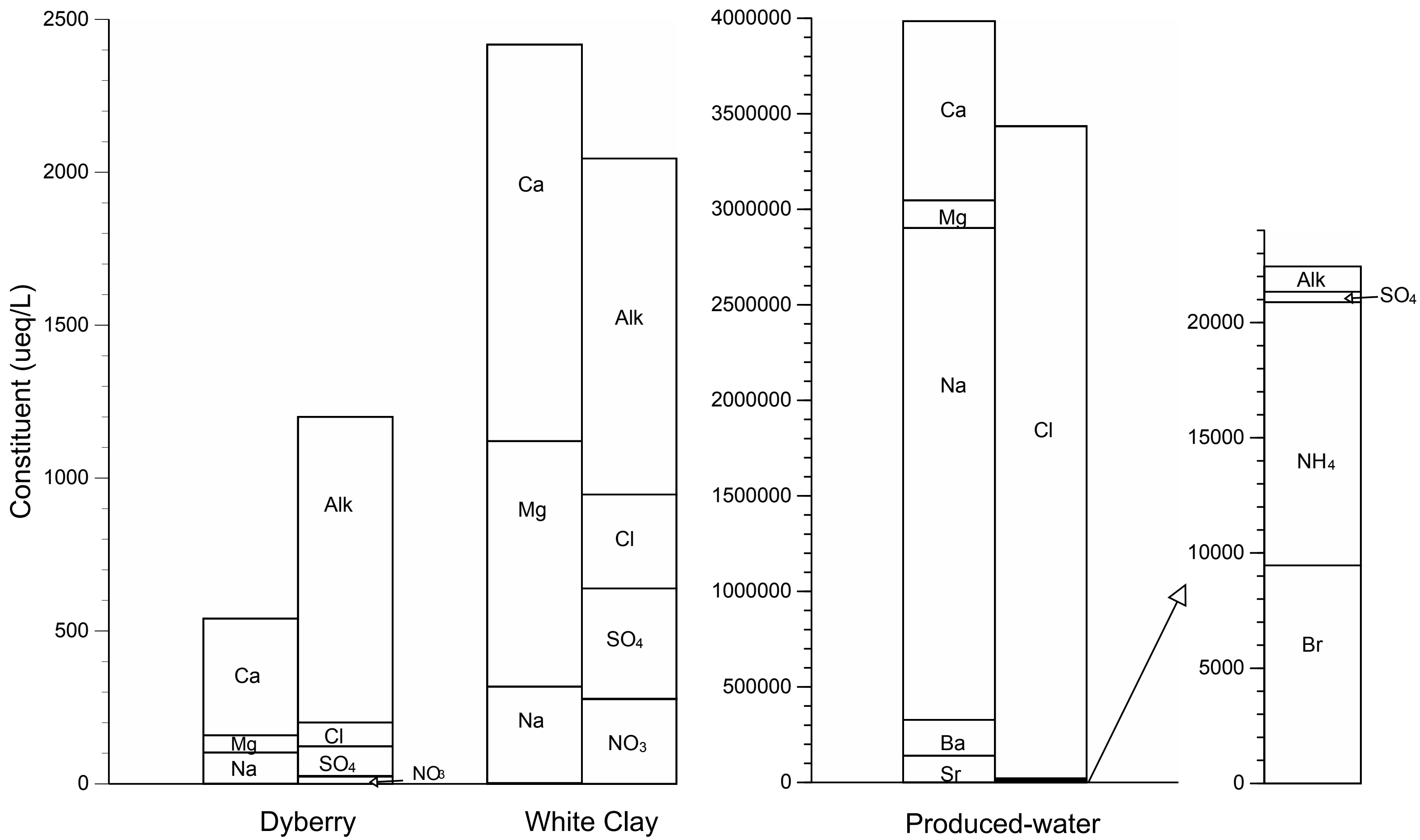


Figure 2. Ion concentration for Dyberry (soft water) and White Clay (hard water) from ambient test, and produced-water sample used in Expt. 4 and 5. Minor anions and cations (e.g., Fe, K, Mn, etc.) not shown. Different scale for Dyberry and White Clay waters versus produced-water. Barium and Strontium were measured in all waters but made up negligible fraction in Dyberry and White Clay waters. The difference between cations and anions for Dyberry is likely the result of an abnormally high alkalinity reading (50 mg/L); typically this stream had alkalinity in the range of 2 to 31 mg/L for other study dates (see Appendices 1-3). Major anions other than chloride shown separately for produced-water.

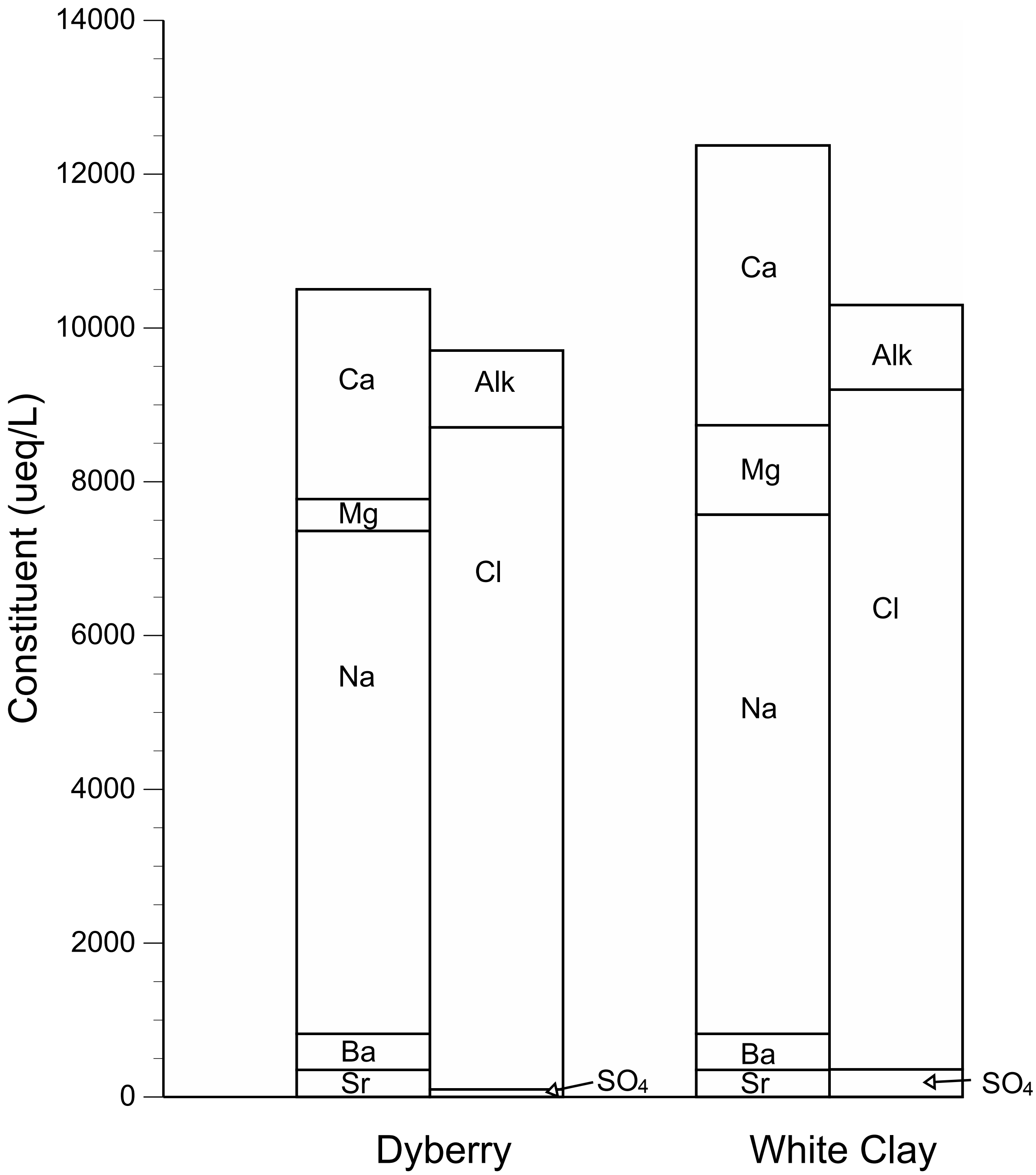


Figure 3. Calculated ion concentration for Dyberry and White Clay waters combined with produced-water at a concentration of 0.25%. Minor cations and anions (e.g., Fe, Mn, K, NH_4, NO_3, Br) are at low concentrations and not shown.

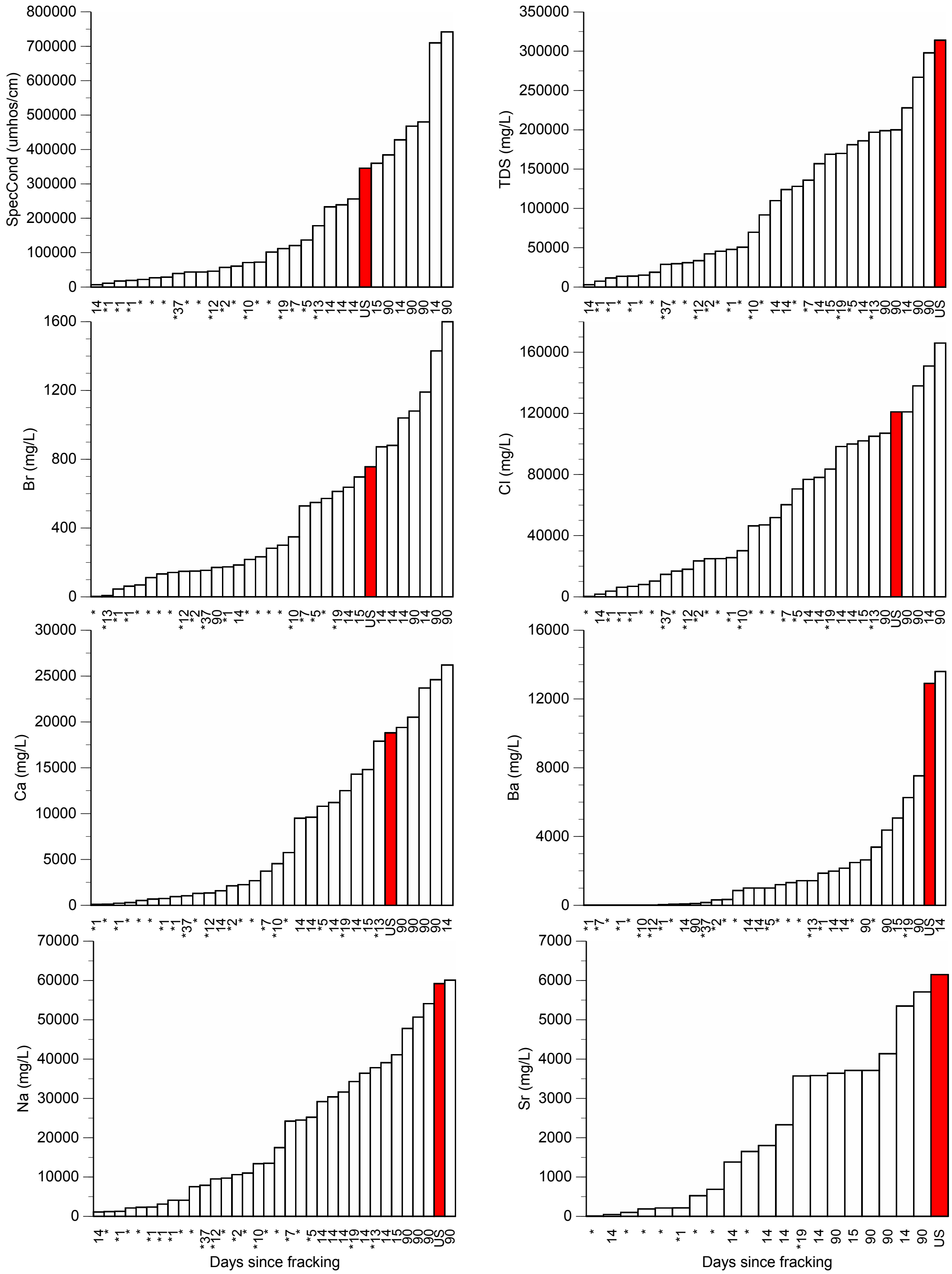


Figure 4. Data on production water from 30 gas wells in Marcellus Shale region. Data from this study indicated as solid bars and as 'US' on x-axis. Asterisks indicate data from Haluszczak et al. (2013); they sampled 22 wells but only had information on when hydrofracking had started for 12 sites. All other results are from Hayes (2009) who sampled 7 horizontal wells 14d and 90d after fracking began (i.e., some wells are represented twice).

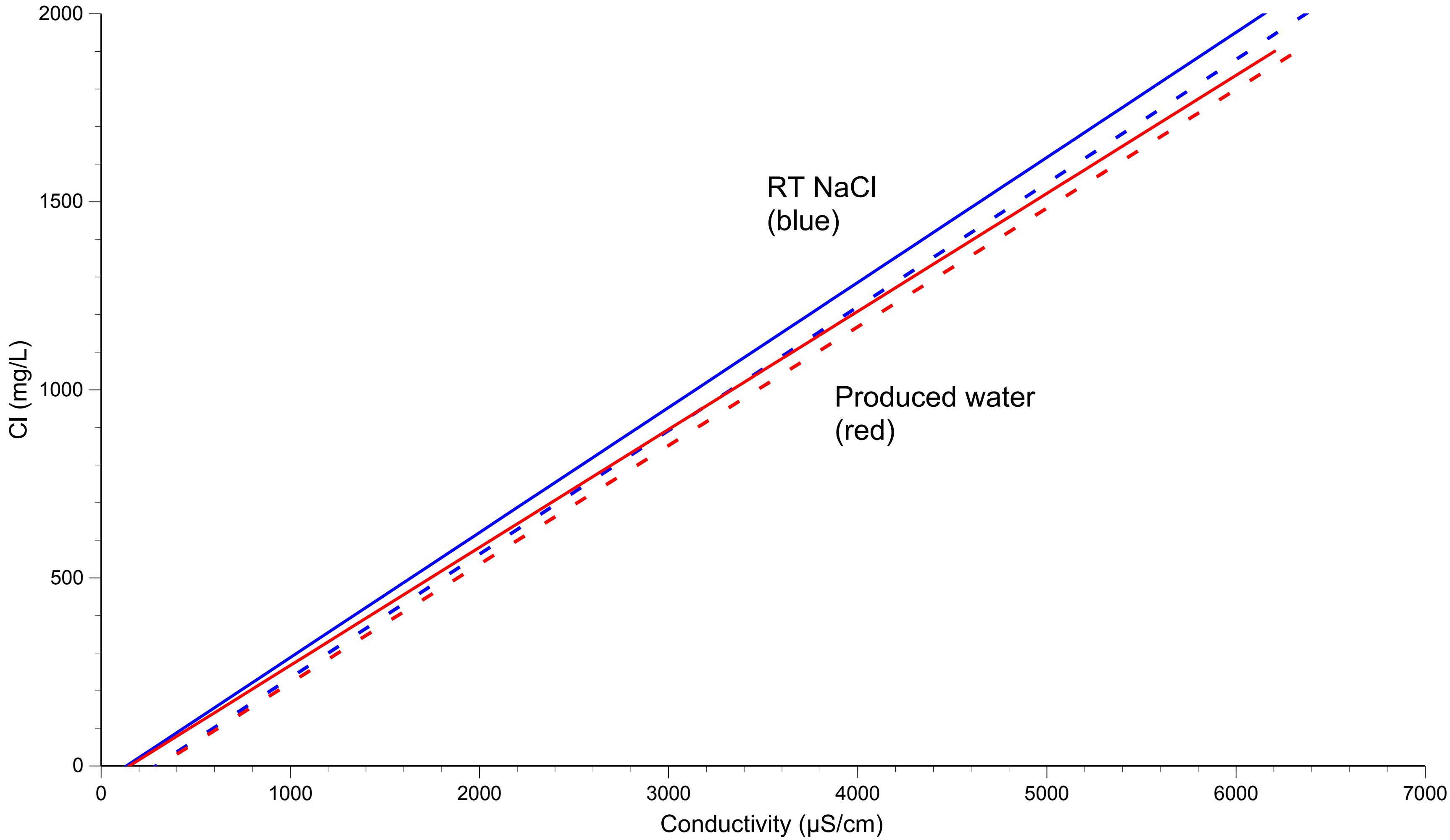


Figure 5. The relationship shown between conductivity and chloride for RT NaCl treatment (blue lines) and produced-water (red lines). Dyberry water is indicated by solid lines and White Clay water by dashes. Chloride levels for produced-water were estimated from the undiluted concentration.

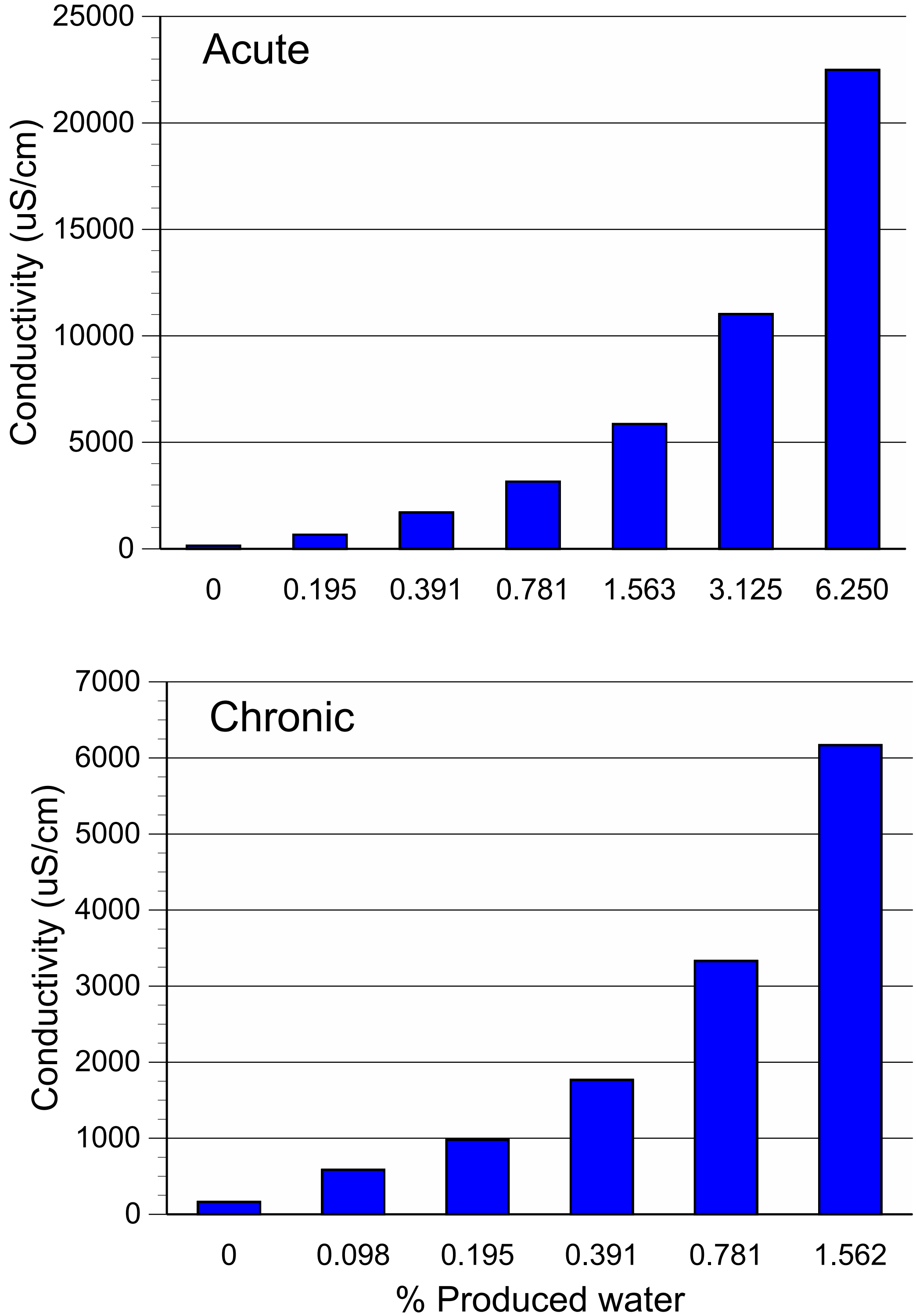


Figure 6. Conductivity shown for five dilutions of the produced-water for the acute and chronic experiments. Data for Dyberry and White Clay water are combined.

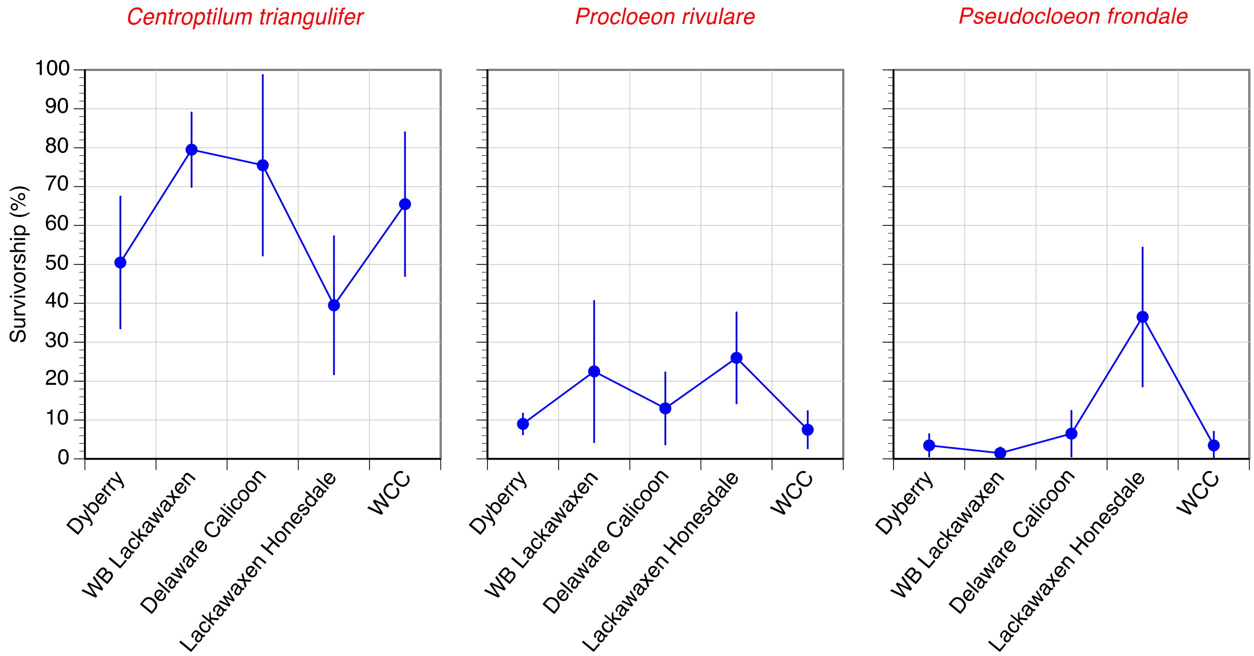


Figure 7. Chronic survivorship (1st instar to adult) of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* in four ambient waters from the upper Delaware River, and White Clay Creek at the Stroud Water Research Center.

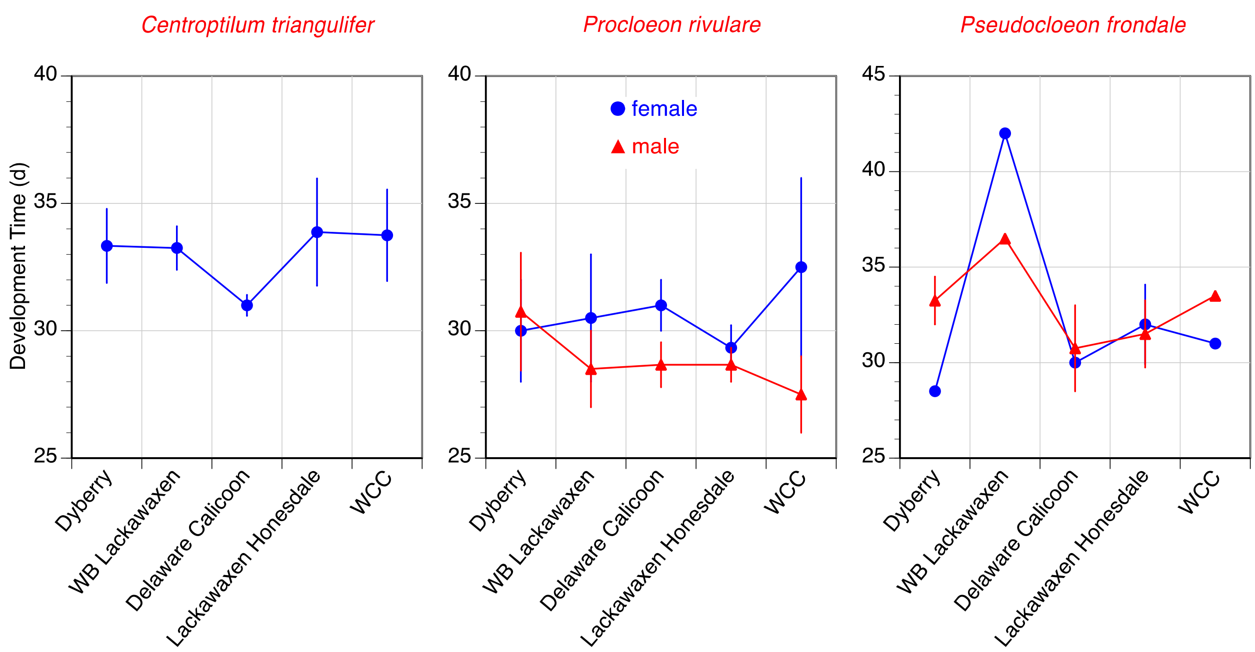


Figure 8. Development time (median days from 1st instar to adult) of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* in four ambient waters from the upper Delaware River, and White Clay Creek at the Stroud Water Research Center.

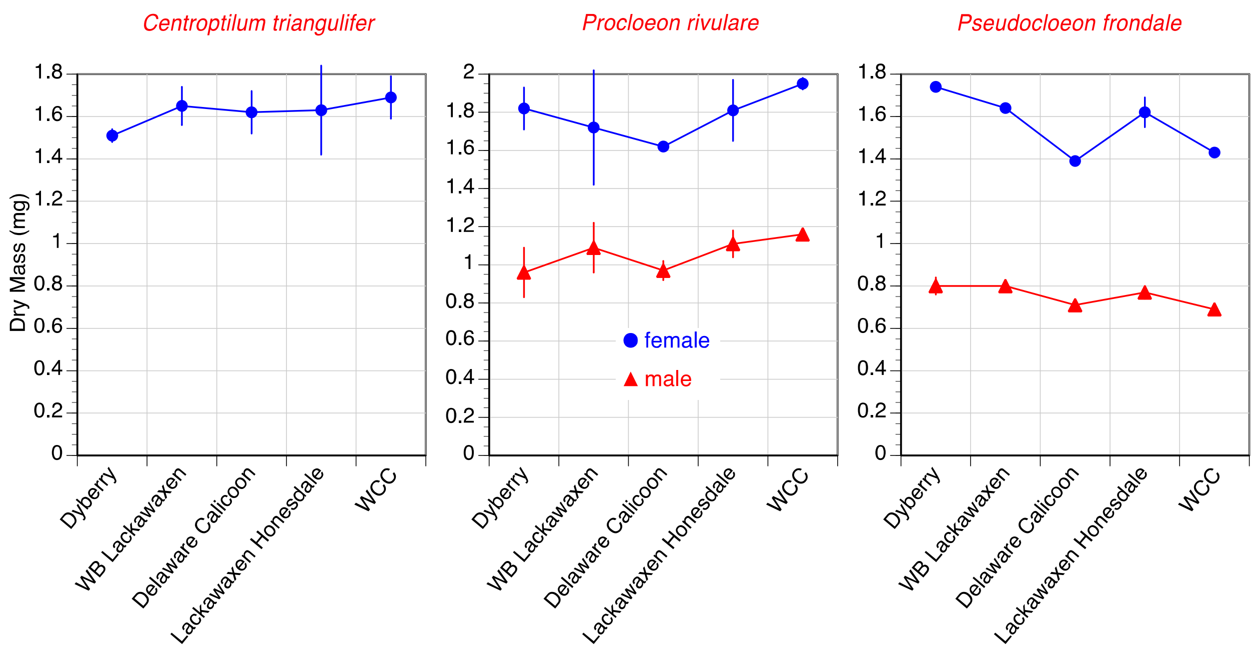
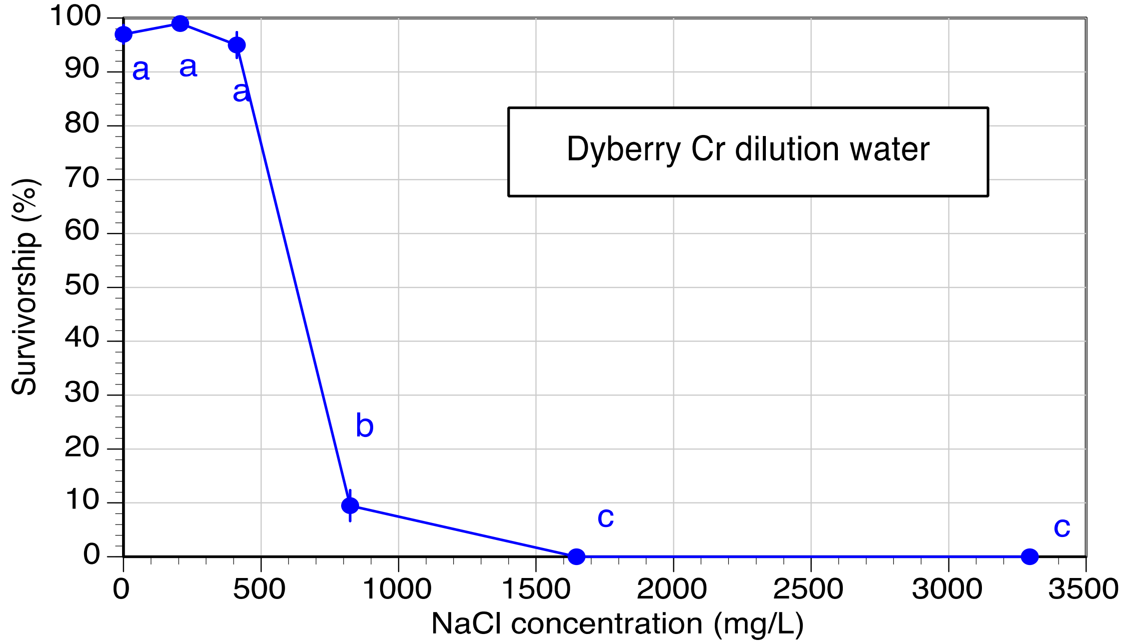


Figure 9. Adult body size (mg dry mass) of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* in four ambient waters from the upper Delaware River, and White Clay Creek at the Stroud Water Research Center.

Centroptilum triangulifer (Clone WCC-2)



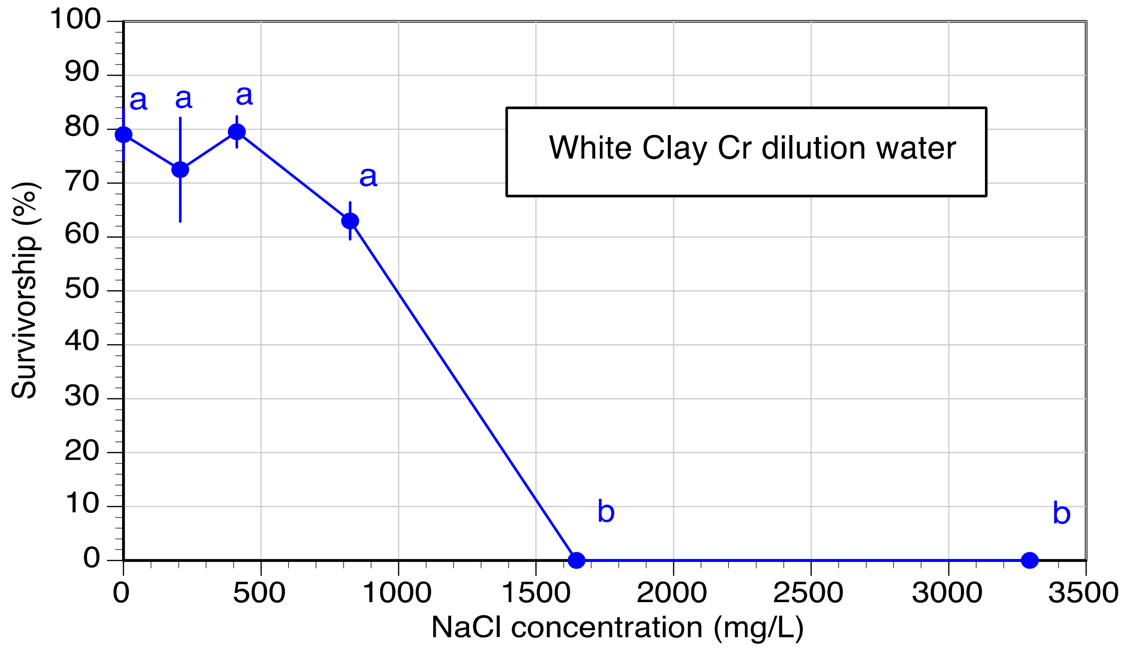


Figure 10. Chronic survivorship (1st instar to adult, %) of *Centroptilum triangulifer* in chronic Reference Toxicant tests using NaCl (0, 206, 412, 824, 1647 and 3295 mg/L) dissolved in water from Dyberry Creek and White Clay Creek.

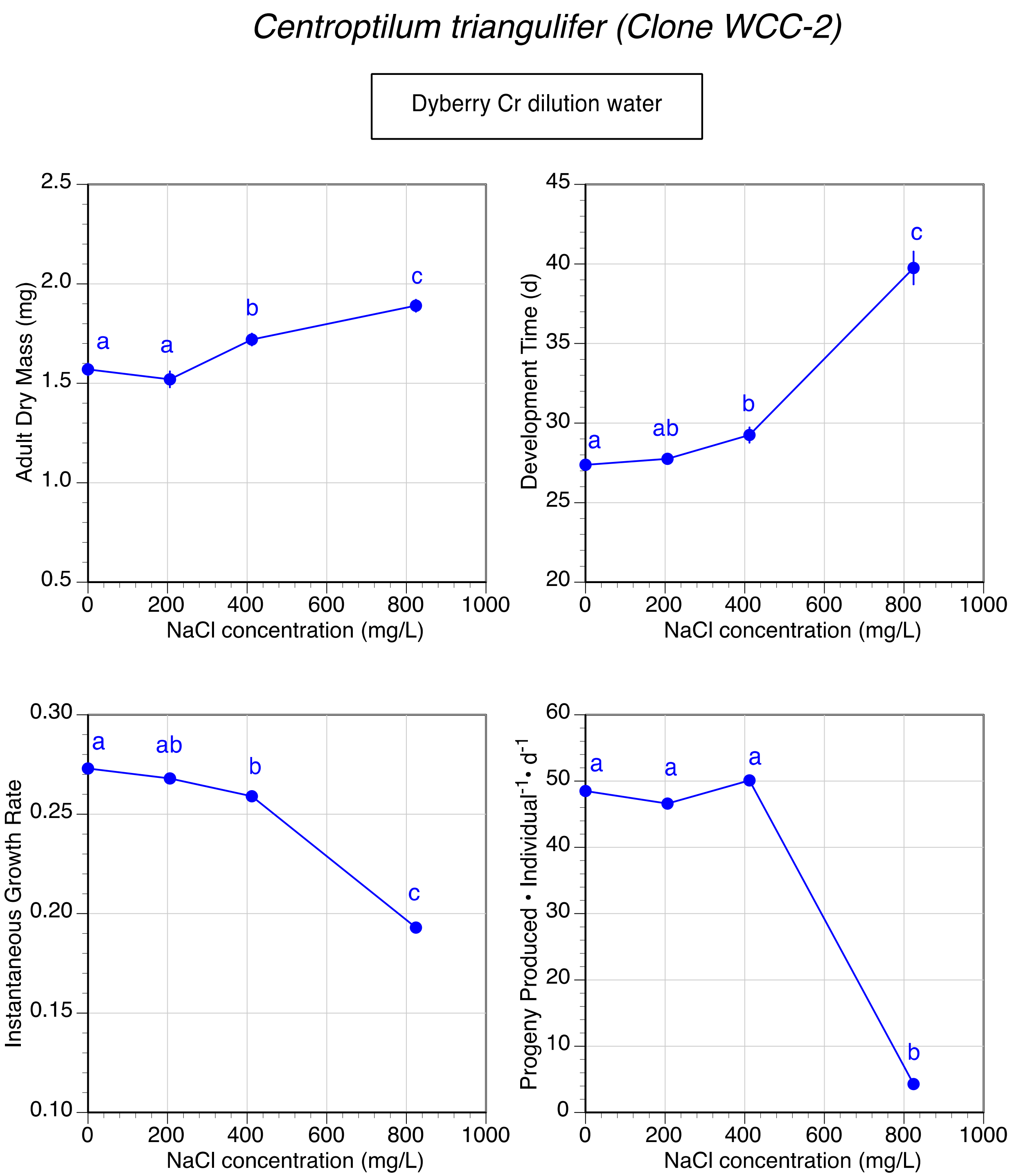


Figure 11. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate and population growth rate (progeny produced per individual per day) of *Centroptilum triangulifer* in chronic Reference Toxicant tests using NaCl (0, 206, 412, 824, 1647 and 3295 mg/L) dissolved in water from Dyberry Creek. There were no survivors in the highest two concentrations.

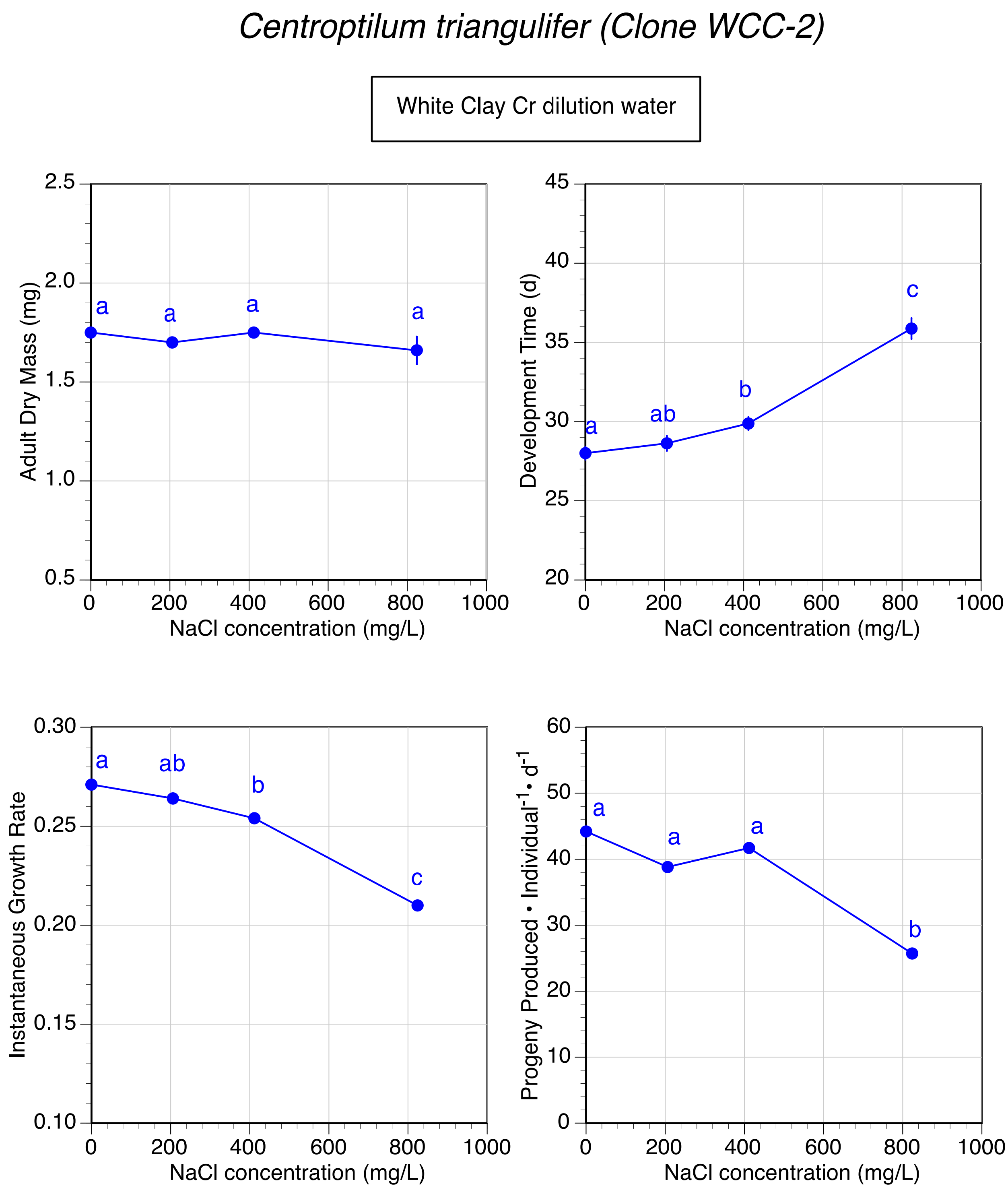


Figure 12. Chronic development time (1st instar to adult, days), adult body size (mg dry mass), instantaneous growth rate and population growth rate (progeny produced per individual per day) of *Centroptilum triangulifer* in chronic Reference Toxicant tests using NaCl (0, 206, 412, 824, 1647 and 3295 mg/L) dissolved in water from White Clay Creek. There were no survivors in the highest two concentrations.

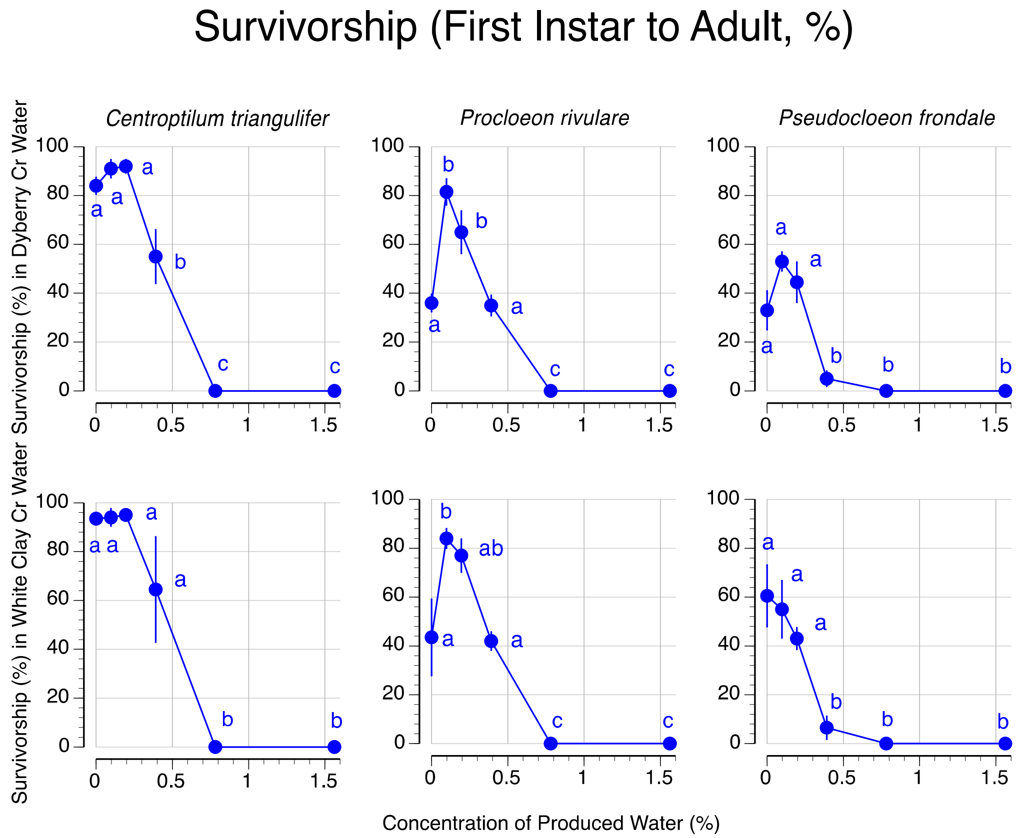


Figure 13. Chronic survivorship (1st instar to adult, %) in full lifecycle exposures of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* to five dilutions of produced-water (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%) dissolved in water from Dyberry Creek and White Clay Creek.

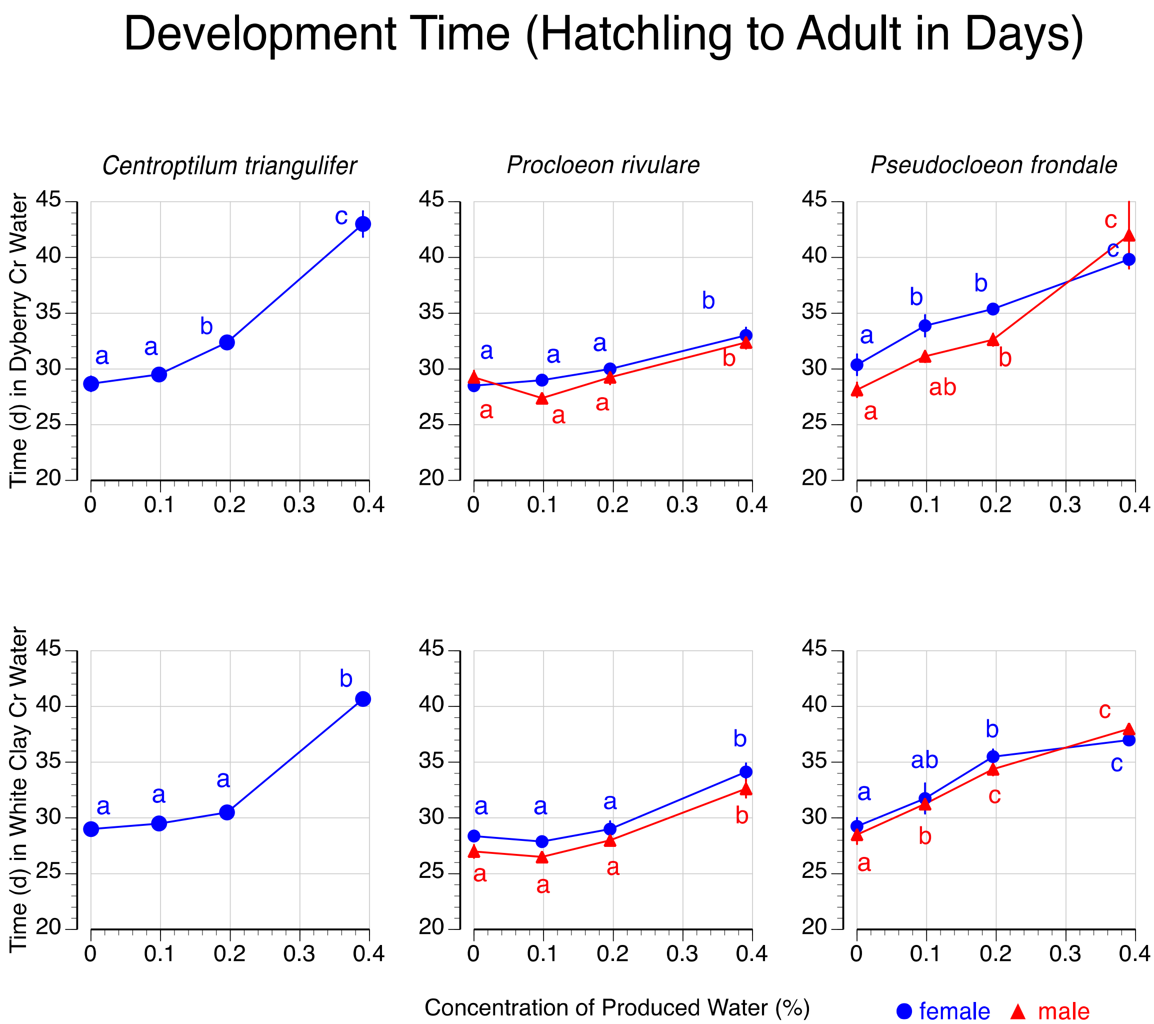


Figure 14. Chronic development time (1st instar to adult, median days) in full lifecycle exposures of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* to five dilutions of produced-water (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%) in water from Dyberry Creek and White Clay Creek. Data from 0.781% and 1.563% are not shown because no larvae survived to the adult stage.

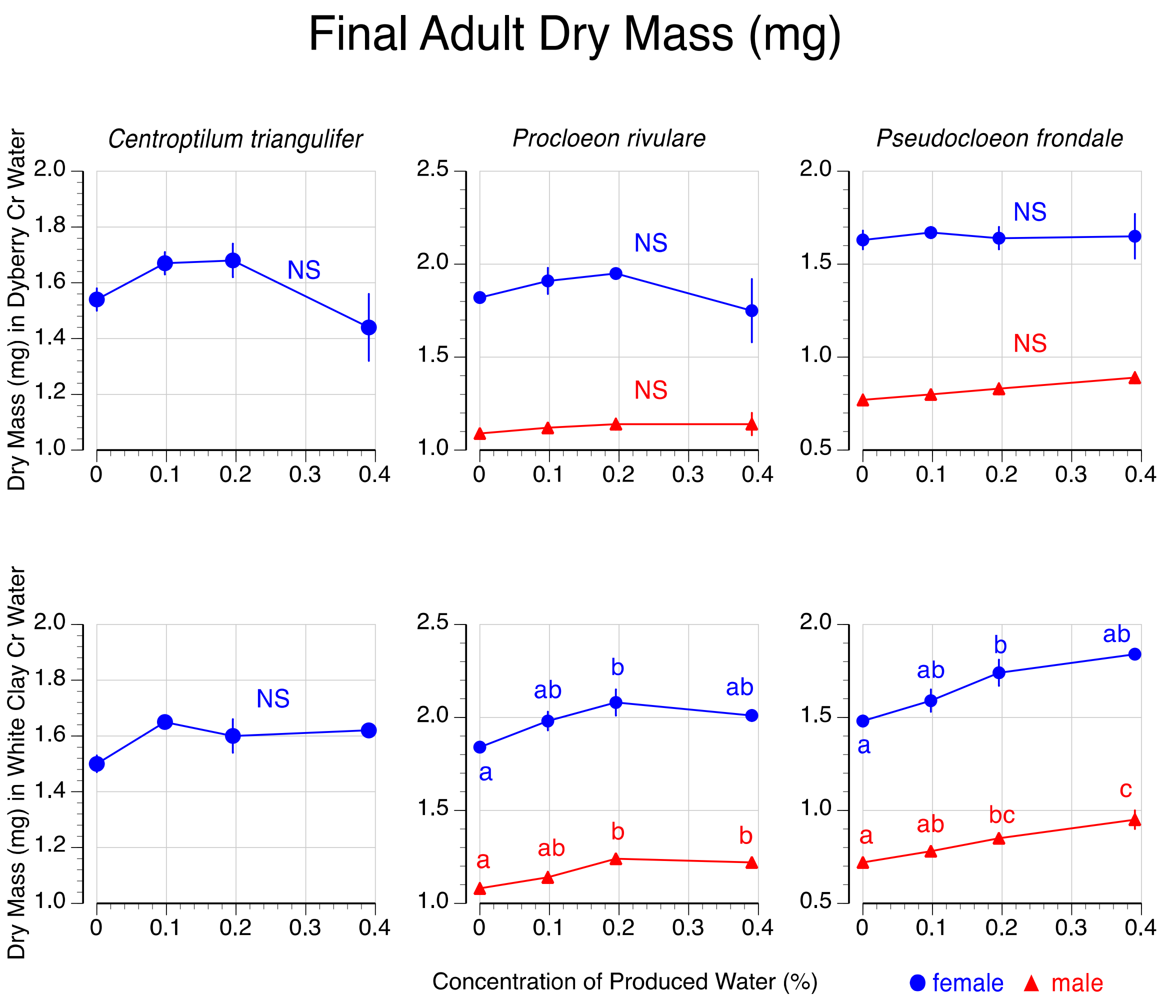


Figure 15. Adult body size (mg dry mass) in full lifecycle exposures of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* to five dilutions of produced-water (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%) in water from Dyberry Creek and White Clay Creek. Data from 0.781% and 1.563% are not shown because no larvae survived to the adult stage.

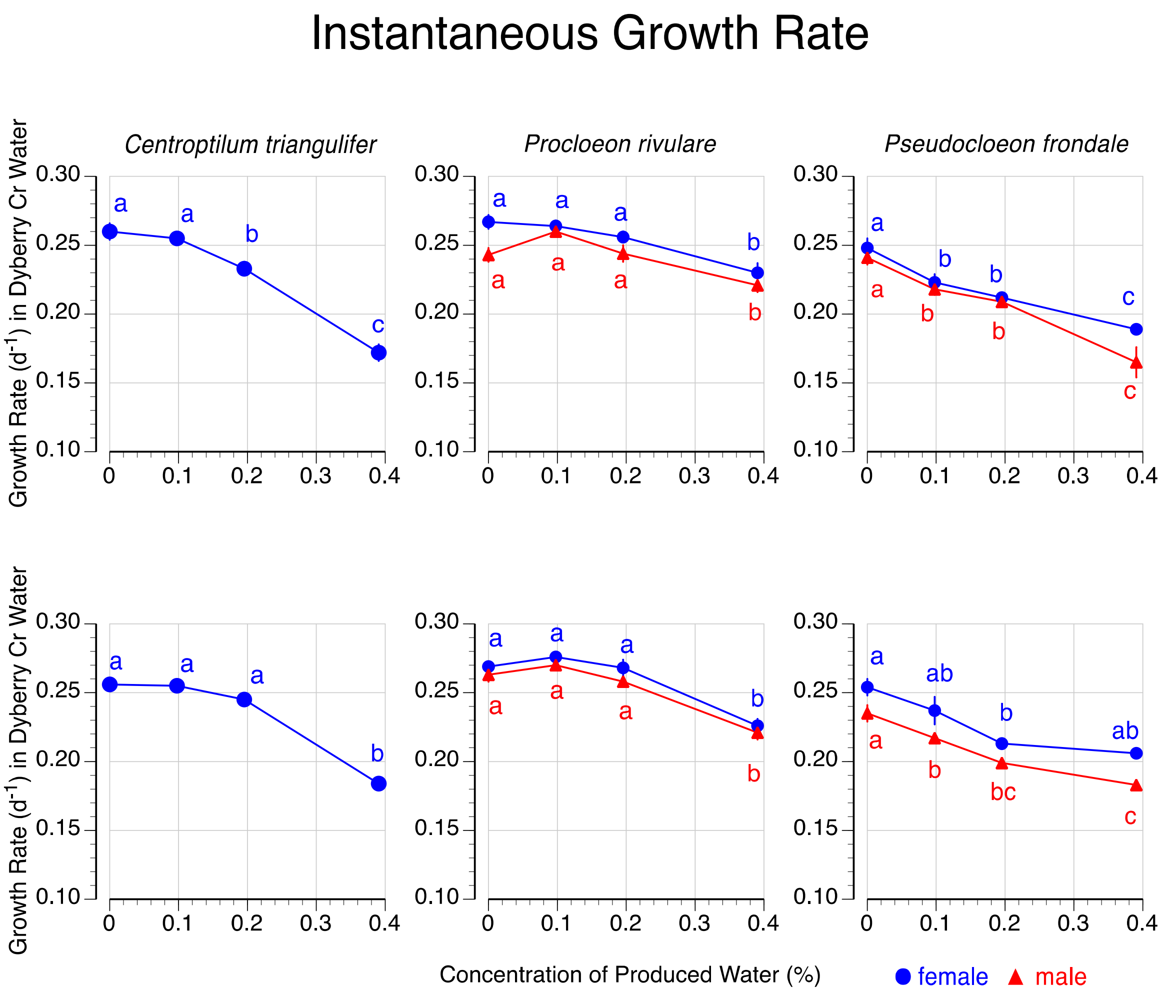


Figure 16. Chronic instantaneous growth rate (1st instar to adult, d^{-1}) in full lifecycle exposures of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* to five dilutions of produced-water (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%) in water from Dyberry Creek and White Clay Creek. Data from 0.781% and 1.563% are not shown because no larvae survived to the adult stage.

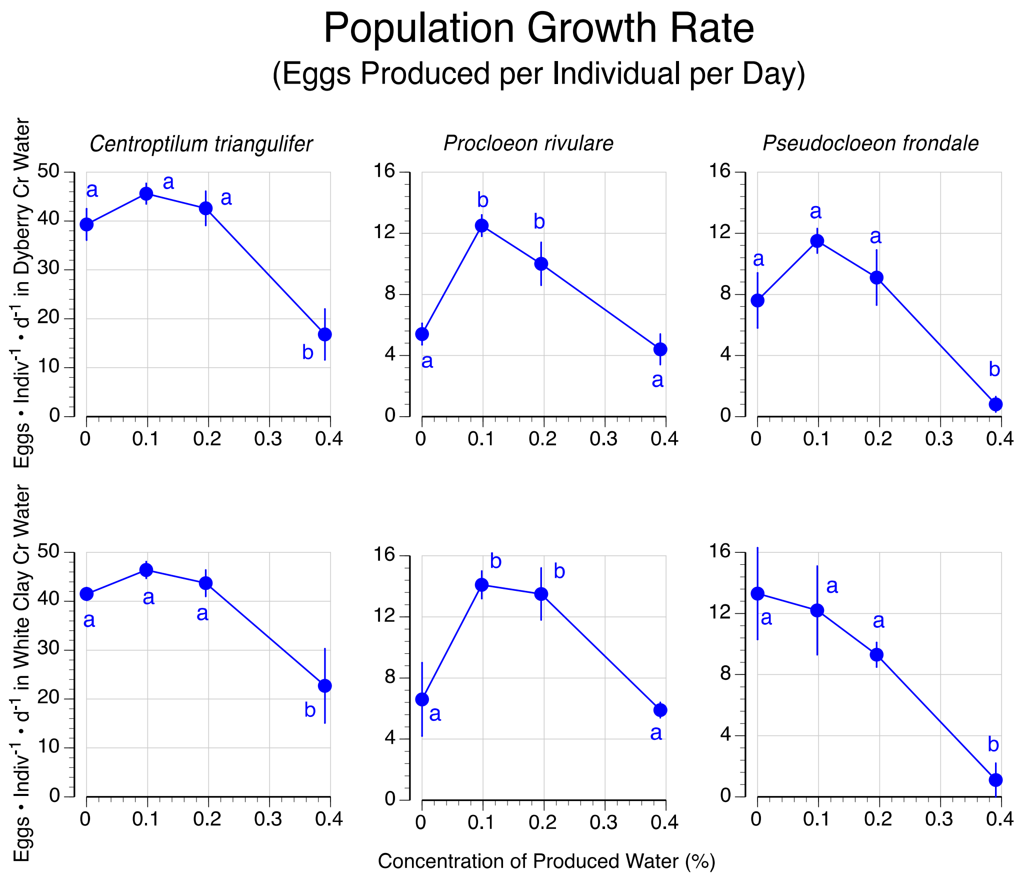


Figure 17. Population growth rate (eggs individual^{-1} d^{-1}) in full lifecycle exposures of *Centroptilum triangulifer*, *Procloeon rivulare*, and *Pseudocloeon frondale* to five dilutions of produced-water (0.0%, 0.098%, 0.195%, 0.391%, 0.781%, and 1.563%) in water from Dyberry Creek and White Clay Creek. Data from 0.781% and 1.563% are not shown because no larvae survived to the adult stage.

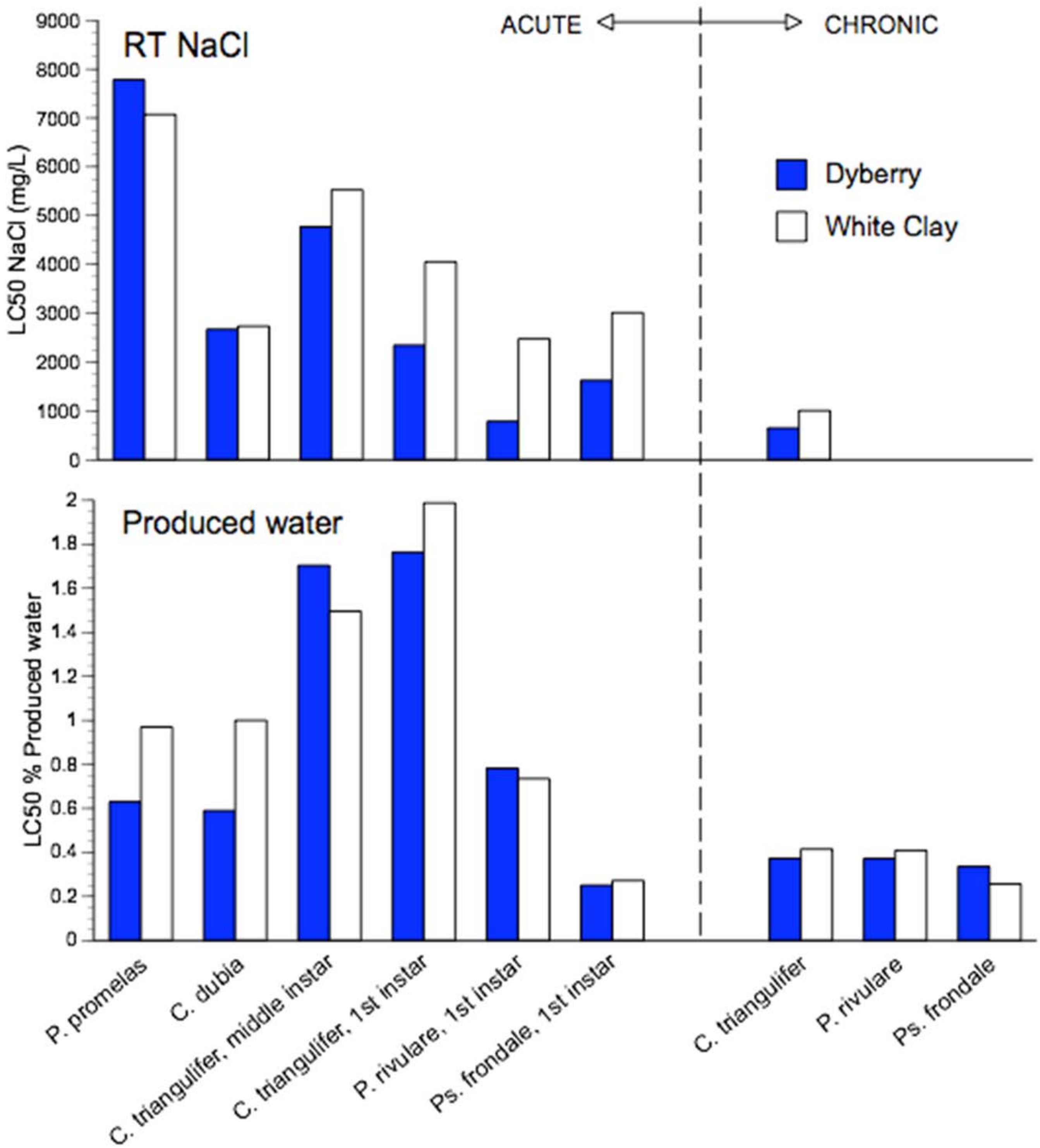


Figure 18. LC50 results for tests involving Reference Toxicant (NaCl) and produced-waters. LC50s are represented as NaCl and as % produced-water depending on test.

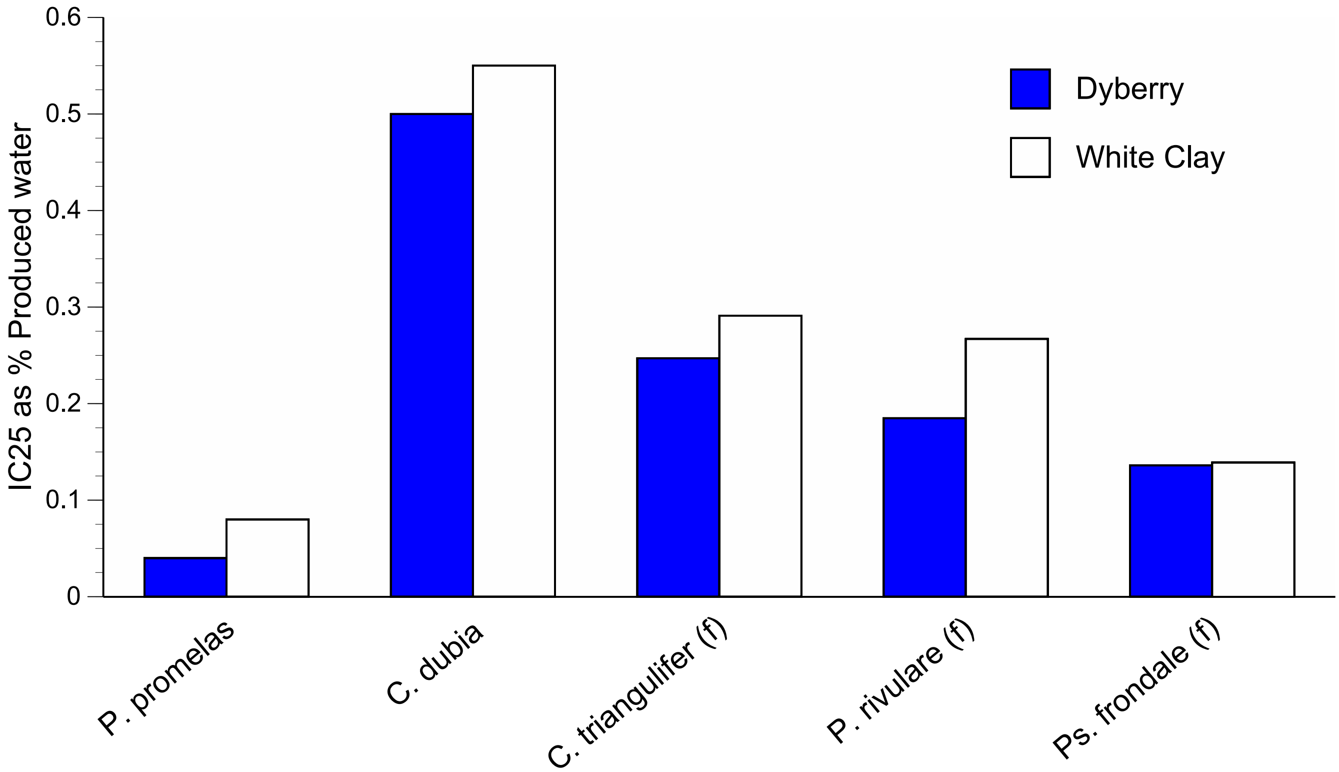


Figure 19. IC25 for chronic toxicity tests of produced-water with *Pimephales promelas* (based on growth), *Ceriodaphnia dubia* (based on reproduction), and mayflies (based on population growth rate). Sex of mayflies was female (f).

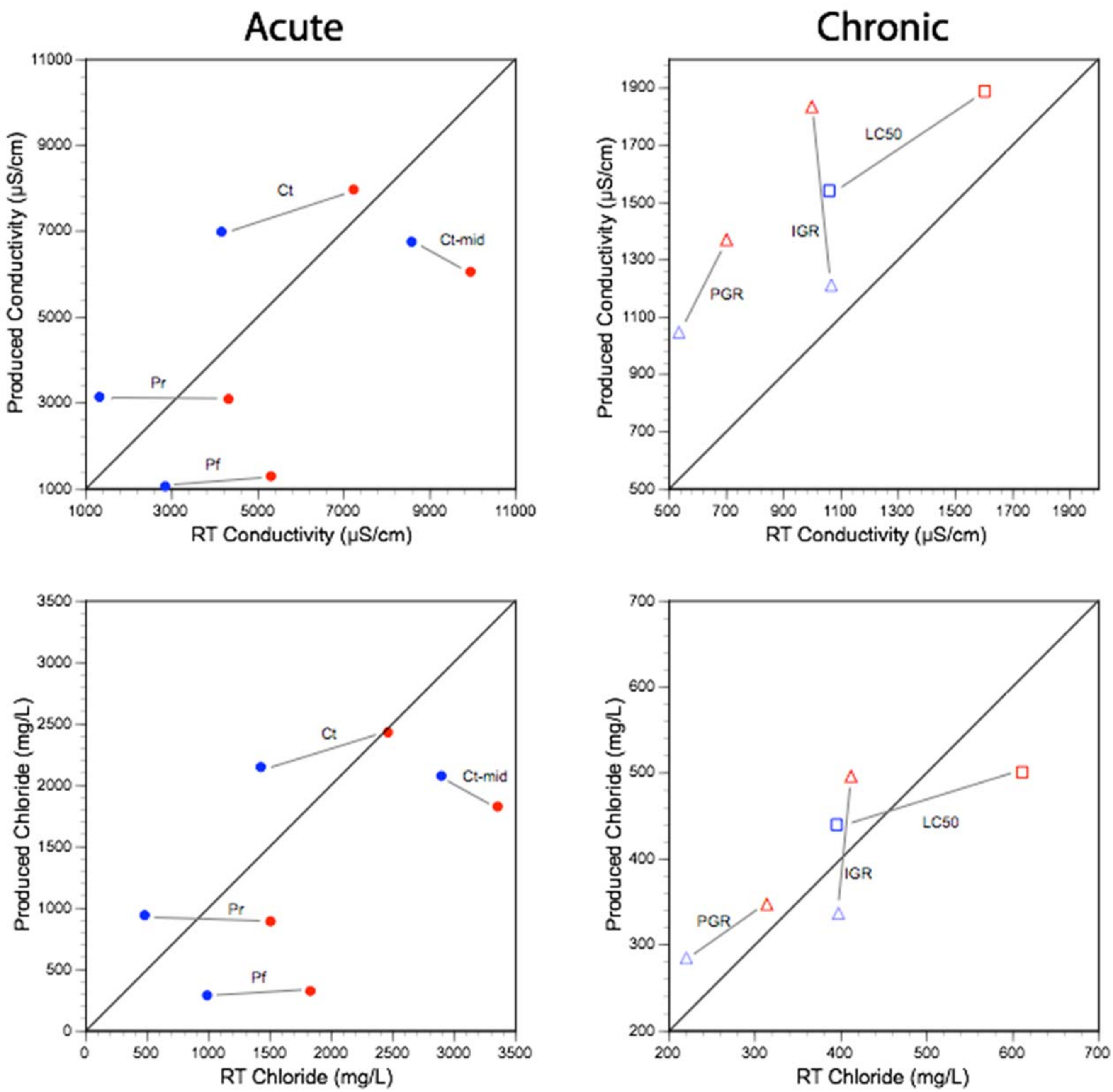


Figure 20. Comparison of reference toxicant (NaCl) and produced-water toxicity to mayflies (using point estimation measures) expressed as specific conductivity or chloride. Solid symbols are acute LC50s (with labels indicating species; all 1st instar except as noted). Open symbols are *C. triangulifer* chronic tests (with labels indicating test type; IGR and PGR are IC25s). Blue symbols are tests in Dyberry Creek dilution water and red are White Clay Creek. Mayfly species are indicated as Ct (*C. triangulifer*, 1st instars), Ct-mid (*C. triangulifer*, middle instars), Pr (*P. rivulare*, 1st instars), and Pf (*Ps. frondale*, 1st instars).

Appendix 1. Water parameters from chronic study on using ambient stream water. Values are from the last day of the experiment [7 May 2012 (*C. triangulifer*), 2 May 2012 (*P. rivulare*), and 1 May 2012 (*Ps. frondale*)]. '<DL' indicates value was below the method detection limit.

Mayfly species	Stream	pH	Temperature (°C)	Conductivity (µS/cm)	% Dissolved oxygen (mg/L)	Ammonia as N (mg/L)	Hardness, Total (mg/L)	Total Alkalinity (mg/L)
Centroptilum triangulifer	Dyberry	7.08	19.8	103	101.9	0.110	35	5
	White Clay	8.09	20.1	258	103.2	0.099	112	62
	Delaware nr. Callicoon	6.51	19.9	120	99.4	0.225	35	<DL
	WB Lackawaxen	7.17	20.1	127	97.4	0.180	44	3
	Lackawaxen nr. Honesdale	7.80	20.1	179	96.1	0.177	72	45
Procloeon rivulare	Dyberry	6.50	20.0	99	104.0	0.142	33	2
	White Clay	8.04	20.1	253	104.4	0.060	108	55
	Delaware nr. Callicoon	6.87	20.0	105	103.8	0.135	33	5
	WB Lackawaxen	7.16	20.1	108	102.3	0.078	37	13
	Lackawaxen nr. Honesdale	7.95	20.1	162	102.6	0.051	65	50
Pseudocloeon frondale	Dyberry	6.92	20.1	86	103.2	0.056	28	2
	White Clay	7.82	20.1	252	101.7	0.100	108	68
	Delaware nr. Callicoon	6.99	20.2	107	103.4	0.514	29	2
	WB Lackawaxen	7.16	20.2	102	103.0	0.058	33	6
	Lackawaxen nr. Honesdale	7.86	20.0	169	101.3	0.060	67	43

Appendix 2. Chronic reference toxicant test using NaCl. Values are based on samples taken at the end of the study (13 Aug 2012), except for initial conductivity measured at the beginning of the study (25 Jun 2012).

Creek	NaCl treatment (mg/L Cl)	pH	Temperature (°C)	Intial Conductivity (µS/cm)	Final Conductivity (µS/cm)	% Dissolved oxygen (mg/L)	Ammonia as N (mg/L)	Hardness, Total (mg/L)	Total Alkalinity (mg/L)
Dyberry	2000	7.56	20	6085	6143	103	0.037	35	30
	1000	7.88	20	3231	3222	106	0.055	36	50
	500	7.94	20	1700	1702	104	0.048	36	42
	250	7.91	20	901	901	104	0.069	39	35
	125	7.92	20	468	506	103	0.057	41	41
	0	7.90	20	73	103	104	0.060	42	32
White Clay	2000	8.38	20	6340	6308	103	0.034	97	45
	1000	8.41	20	3364	3377	105	0.047	99	75
	500	8.42	20	1848	1831	106	0.058	106	69
	250	8.25	20	1062	1053	105	0.064	107	85
	125	8.22	20	657	647	105	0.059	106	80
	0	8.47	20	241	232	107	0.073	106	82

Appendix 3. Chronic test using produced-water. Water was sampled on the final day of test [30 Aug 2012 (*C. triangulifer*), 31 Aug 2012 (*P. rivulare*), and 4 Sept 2012 (*Ps. frondale*)], except for initial conductivity (19 Jul 2012) sampled at the start of the study. '<DL' indicates value was below the method detection limit; detection limit for ammonia was <0.0112 mg/L.

Mayfly species	Creek	Produced water concentration	pH	Temperature (°C)	Initial Conductivity (µS/cm)	Final Conductivity (µS/cm)	Dissolved oxygen (mg/L)	Ammonia as N (mg/L)	Hardness, Total (mg/L)	Total Alkalinity (mg/L)
Centroptilum triangulifer	Dyberry	0.015625	7.73	20	6113	6110	9.26	0.029	941	9
		0.0078125	7.64	20	3255	3287	9.24	0.027	469	22
		0.00390625	8.19	20	1694	1690	9.43	0.027	252	21
		0.001953125	7.67	20	905	928	9.24	0.029	155	9
		0.000976563	7.51	20	509	529	9.16	0.030	101	22
		0	7.89	20	80	117	9.10	0.038	45	24
	White Clay	0.015625	8.13	20	6240	6255	9.18	0.027	1020	43
		0.0078125	8.32	20	3379	3428	9.22	0.033	564	62
		0.00390625	8.43	20	1833	1836	9.45	0.044	319	68
		0.001953125	8.16	20	1046	1057	9.37	0.050	220	55
		0.000976563	8.08	20	662	685	9.25	0.049	172	50
		0	8.10	20	241	275	9.16	0.052	117	62
Procloeon rivulare	Dyberry	0.015625	7.61	20	6120	6160	8.64	<DL	916	10
		0.0078125	7.82	20	3278	3294	8.64	<DL	478	23
		0.00390625	8.09	20	1702	1708	9.03	0.029	249	29
		0.001953125	7.88	20	908	922	8.82	0.022	151	32
		0.000976563	7.91	20	506	523	8.81	0.024	100	41
		0	8.06	20	81	121	8.57	0.031	45	31
	White Clay	0.015625	8.36	20	6180	6183	8.92	<DL	982	51
		0.0078125	8.67	20	3410	3390	9.43	<DL	534	60
		0.00390625	8.77	20	1833	1808	9.31	<DL	307	64
		0.001953125	8.63	20	1045	1030	9.11	<DL	210	77
		0.000976563	8.60	20	658	658	9.14	<DL	165	72
		0	8.27	20	241	259	9.08	0.027	111	68
Pseudocloeon frondale	Dyberry	0.015625	7.62	20	6098	6163	9.01	<DL	916	7
		0.0078125	7.77	20	3249	3272	9.12	<DL	469	16
		0.00390625	7.79	20	1700	1704	9.09	<DL	239	25
		0.001953125	8.32	20	914	921	9.12	<DL	143	24
		0.000976563	7.75	20	503	511	9.18	<DL	92	32
		0	7.62	20	83	105	9.13	<DL	42	8
	White Clay	0.015625	8.03	20	6265	6255	8.85	0.026	951	52
		0.0078125	8.47	20	3412	3399	9.10	<DL	540	60
		0.00390625	8.24	20	1837	1830	9.11	<DL	315	58
		0.001953125	8.29	20	1053	1039	9.14	<DL	211	69
		0.000976563	8.28	20	665	662	9.04	<DL	160	55
		0	8.23	20	243	255	8.98	0.022	107	61

Appendix 4. Data on production water from 30 gas wells in Marcellus Shale region. Seven wells are from horizontal wells after 14 and 90 days after fracturing and parameters are based on dissolved measurements (Hayes 2009). For 12 wells days since fracturing ranged from 1-37 days, with the remaining wells not having this information available (Haluszczak et al. 2013).

County, State	Site ID.	days after fracking	SpCond (umhos/cm)	TDS (mg/L)	pH (pH units)	Alkalinity (mg/L $CaCO_3$)	SO_4 (mg/L)	Br (mg/L)	Cl (mg/L)	Ca (mg/L)	Ba (mg/L)	Mn (ug/L)	K (mg/L)	Na (mg/L)	Sr (mg/L)	Study
Washington, PA	4	10	71,200	69,700	6.6	143	226	349	30,200	4540	10	1620	161	13,400	–	Haluszczak et al. 2013
Butler, PA	7	na	–	–	–	–	59	572	51,800	–	7	3000	4	24,500	–	Haluszczak et al. 2013
Washington, PA	10	7	120,600	136,000	6.4	130	49	528	60,200	3730	4	3450	337	24,200	–	Haluszczak et al. 2013
Butler, PA	11	37	39,200	28,900	7.6	273	25	154	14,700	1040	161	553	68	7910	–	Haluszczak et al. 2013
Huntington, PA	12	12	46,200	33,700	5.9	309	51	148	18,000	1350	15	29,400	40	9530	–	Haluszczak et al. 2013
Susquehanna, PA	16	1	–	47,800	7.4	223	<5	174	25,600	100	1870	970	57	1280	–	Haluszczak et al. 2013
Susquehanna, PA	17	na	–	50,700	7.7	241	<5	142	24,900	112	1430	1110	54	1210	–	Haluszczak et al. 2013
Clearfield, PA	20	1	10,990	7520	7.5	238	30	45	3700	230	40	670	20	2350	–	Haluszczak et al. 2013
Clearfield, PA	21	5	137,000	181,000	6.3	112	7	548	70,600	10,800	1010	3030	616	25,200	–	Haluszczak et al. 2013
Clearfield, PA	22	13	178,200	197,000	6.1	106	<5	8	105,000	17,900	1430	3260	890	37,800	–	Haluszczak et al. 2013
Somerset, PA	23	2	57,100	42,200	7.0	298	15	150	23,500	2120	314	2420	79	10,600	–	Haluszczak et al. 2013
Clearfield, PA	25	na	26,700	15,100	11.6	607	384	300	8010	–	3380	2200	5240	2110	–	Haluszczak et al. 2013
Greene, PA	26	1	19,240	13,900	5.1	29	296	62	6900	730	4	2330	95	3090	–	Haluszczak et al. 2013
Clearfield, PA	27	na	21,900	13,700	10.8	939	420	<2	390	675	2490	3130	3560	2280	6	Haluszczak et al. 2013
Fayette, PA	28	1	17,650	11,600	7.8	172	279	–	6200	949	9	535	161	4090	217	Haluszczak et al. 2013
Fayette, PA	30	na	60,500	45,600	7.2	199	394	233	25,000	2680	9	497	421	11,000	527	Haluszczak et al. 2013
Fayette, PA	31	na	43,700	29,700	6.9	165	16	133	–	1300	75	1670	67	9730	187	Haluszczak et al. 2013
Clinton, PA	32	19	>111,900	170,000	6.3	235	<5	613	83,500	12,500	6270	4990	224	34,300	3570	Haluszczak et al. 2013
Indiana, PA	35	na	101,700	128,000	6.9	152	11	283	46,400	5750	1320	7400	215	17,500	1650	Haluszczak et al. 2013
Clinton, PA	39	na	72,200	91,800	7.6	247	<5	217	47,000	2240	1200	800	66	13,500	687	Haluszczak et al. 2013
Susquehanna, PA	40	na	28,900	18,800	7.7	212	<5	69	10,300	291	340	280	25	4100	99	Haluszczak et al. 2013
Susquehanna, PA	41	na	43,600	31,000	–	–	<5	112	16,800	523	861	780	50	7540	213	Haluszczak et al. 2013
Lewis, WV	C	14	239,000	110,000	6.1	71	22	637	78,100	9500	2160	4420	533	29,200	1800	Hayes 2009
Lewis, WV	C	90	384,000	267,000	5.9	41	50	1080	107,000	19,400	4370	9900	548	54,100	3710	Hayes 2009
Greene, PA	E	14	256,000	124,000	6.0	88	40	880	76,800	9600	1010	5590	299	31,600	1380	Hayes 2009
Greene, PA	E	90	468,000	199,000	5.9	40	42	1430	121,000	23,700	2640	12,800	782	60,100	3640	Hayes 2009
Washington, PA	F	14	233,000	157,000	6.2	60	89	1040	100,000	14,300	76	7740	394	36,400	2330	Hayes 2009
Washington, PA	F	90	480,000	200,000	5.9	12	33	1600	138,000	24,600	104	11,000	461	47,800	4140	Hayes 2009
Lycoming, PA	G	15	360,000	169,000	6.4	48	49	697	102,000	14,800	5070	5170	274	41,100	3710	Hayes 2009
Lycoming, PA	G	90	742,000	298,000	5.8	25	100	171	166,000	20,500	7530	8760	477	50,700	5710	Hayes 2009
Bradford, PA	K	14	6800	3010	6.9	91	7	185	1670	1590	1010	1230	8	1100	46	Hayes 2009
McKean, PA	M	14	710,000	228,000	5.8	26	50	1190	151,000	26,200	1990	8420	1010	30,400	5350	Hayes 2009
Susquehanna, PA	O	14	428,000	186,000	6.6	95	50	872	98,300	11,200	13,600	7630	253	39,100	3580	Hayes 2009
?, PA - composite production water		na	345,600	314,000	4.9	55	22	756	121,000	18,800	12,900	6880	351	59,200	6150	This study