samples the effluents tended to have lower pH values and higher DOC and ion concentrations. The variation in pH, DOC, and ion concentrations show in these two datasets are consistent with the values seen in detailed sample analyses reported in Table 1.

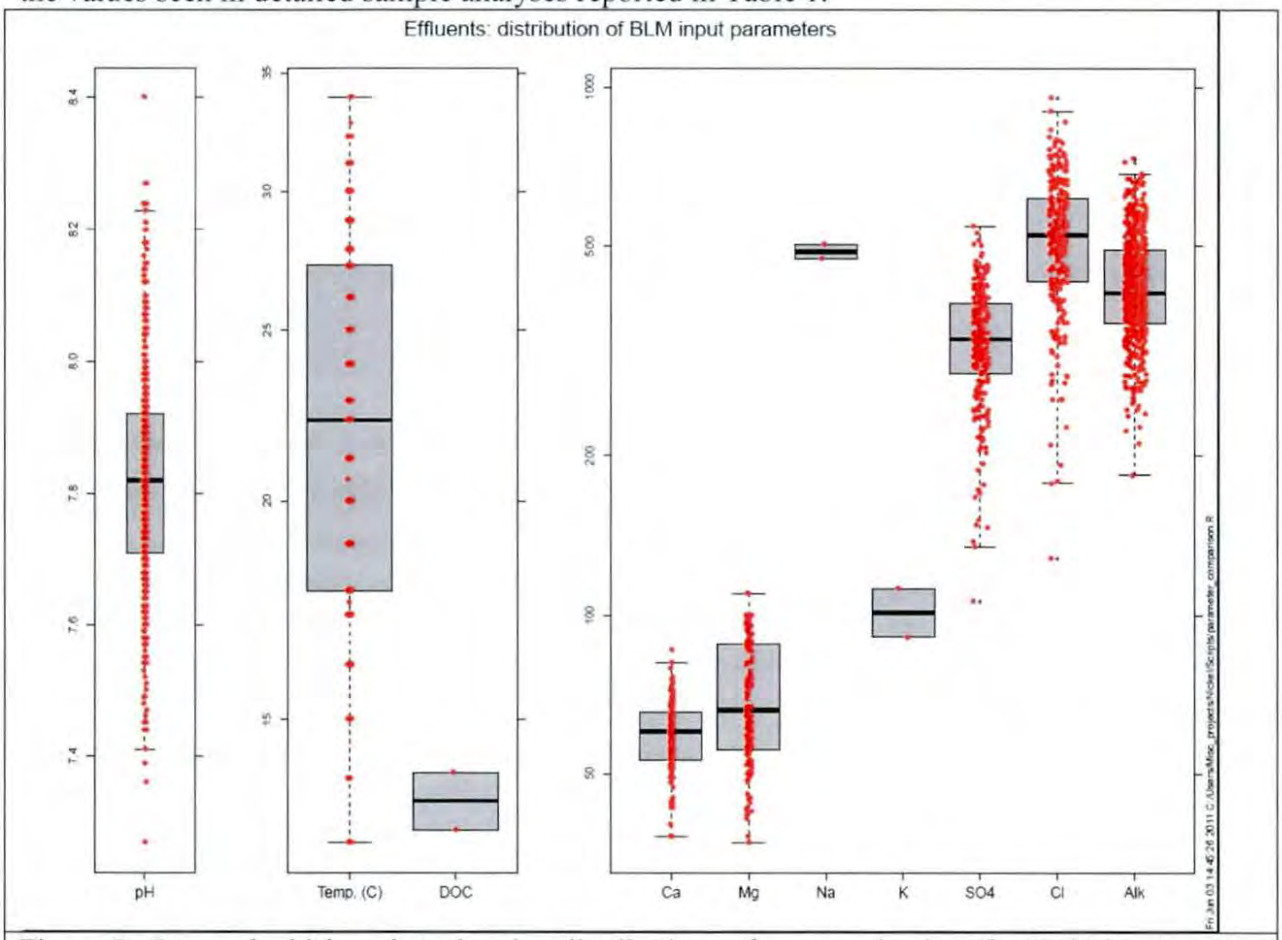


Figure 5. Box and whisker plots showing distributions of measured values for BLM input parameters in effluent samples. Average values are shown by a black line in the middle of each box and represent mean (pH, Temp, DOC) or geometric mean (Ca, Mg, Na, K, SO4, Cl, Alk) depending on whether parameters are expected to be normally or log-normally distributed. For each box, the lower edge of the box represents the 25th percentile, the upper edge of the box represents the 75th percentile, and whiskers extend to minimum and maximum values exclusive of extreme values. Individual observations are shown as small red circles.

Variability in BLM input parameters was used in a sensitivity analysis to determine the degree to which predicted toxicity may be expected to change over time. The model was first run for a base case that used median values for all parameters shown in Figure 4 and Table 3.

For each BLM parameter, two additional runs were then performed by substituting either

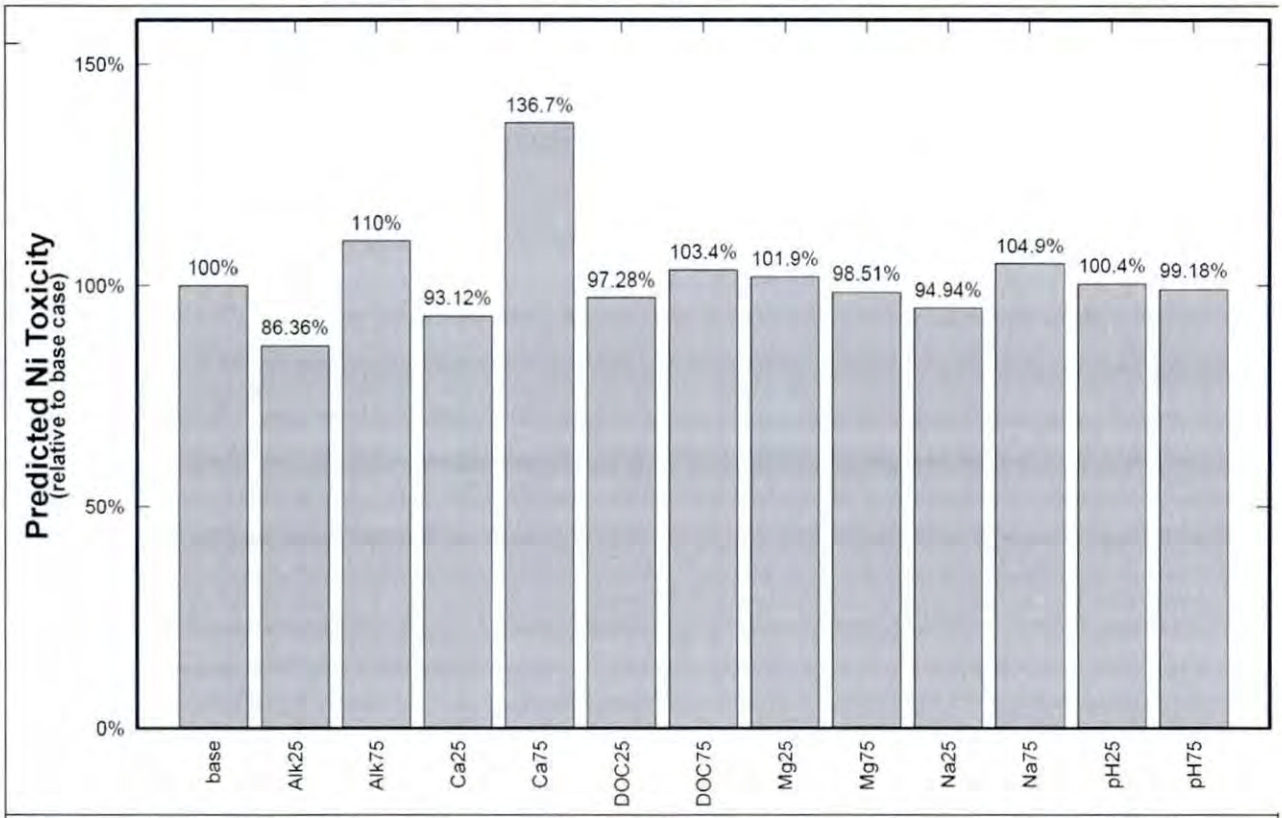


Figure 6. Sensitivity analysis of varying input parameters to the BLM on predicted Nickel toxicity in river samples. For the base case, average values for all parameters shown in Figure 4 were used. A series of additional simulations were then run to see the effect of variation in individual parameter values on the base case. For each additional simulation, the base case was modified with either the 25th or the 75th percentile value of an input variable, while all other parameters were held at the values used for the base case. For example, the result labeled "Alk25" uses the 25th percentile for alkalinity (shown in Figure 4), and the result "Alk75" uses the 75th percentile for alkalinity. Sensitivity results for other parameters are labeled with a similar labeling scheme.

the 25% or 75% value from the box and whisker plots in Figure 4 for the average value, while keeping all other parameters constant, at their respective average. The resulting sensitivity analyses are shown in Figure 6 for river samples considering variation at the 25th and 75th percentile, and Figure 7 considering variation at the 10th and 90th percentiles.

Variation in input values at the 25th and 75th percentiles for river water samples had relatively little effect on the predicted Nickel toxicity, with the largest effects resulting from

changes in alkalinity and calcium concentrations. A similar pattern was observed when variation at the 10^{th} and 90^{th} percentiles were considered (Figure 7). Even at these extreme values, the expected variation in predicted nickel toxicity ranges from about 70 to 150 percent of the base case value. Guidance for derivation of site-specific adjustments to water quality criteria based on the WER procedure allow simple geometric means of individual WER values when the range in values is within a factor of 5. Since the effects of the variation in river water chemistry on nickel toxicity will be well within that those limits, this uncertainty analysis supports the conclusion that average conditions from a relatively small number of samples should provide an acceptable characterization for deriving a site-specific nickel criterion. As a result of these sensitivity analyses, the calculated WER for the site is not expected to significantly change as a result of variability in water quality within ranges comparable to these existing monitoring datasets.

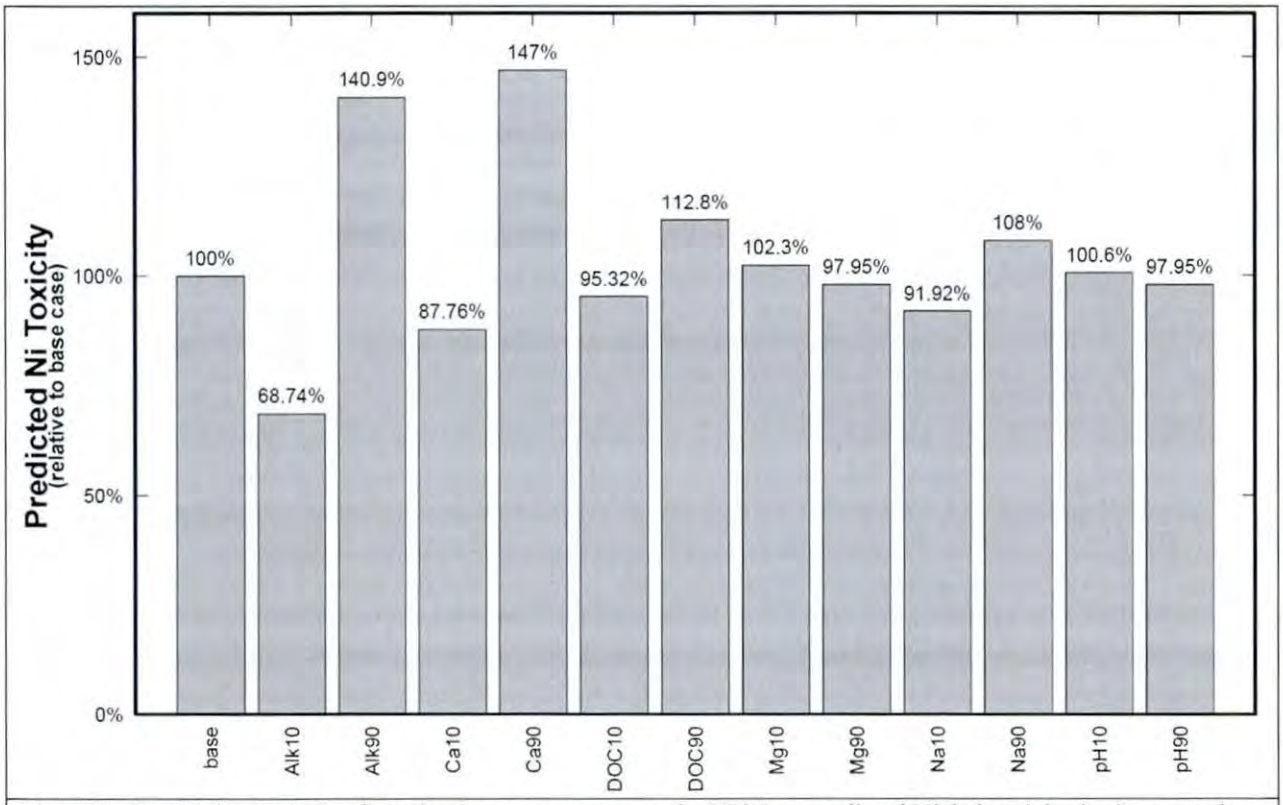


Figure 7. Sensitivity analysis of varying input parameters to the BLM on predicted Nickel toxicity in river samples. For the base case, average values for all parameters shown in Figure 4 were used. A series of additional simulations were then run to see the effect of variation in individual parameter values on the base case. For each additional simulation, the base case was modified with either the 10^{th} or the 90^{th} percentile value of an input variable, while all other parameters were held at the values used for the base case. For example, the result labeled "Alk10" uses the 10^{th} percentile for alkalinity (shown in Figure 4), and the result "Alk90" uses the 90^{th} percentile for alkalinity. Sensitivity results for other parameters are labeled with a similar labeling scheme.

For effluent samples (Figure 8), variation in alkalinity had the largest effect on predicted nickel toxicity. However, the resulting variation in predicted LC50 values was small, corresponding to a little more than 10% change relative to the base case. Variation in effluent characteristics is only presented for comparison to that seen for river water, since it is only the downstream river water that will be used to estimate the site-specific nickel adjustment.

Figure 8. Sensitivity analysis of varying input parameters to the BLM on predicted Nickel toxicity in effluent samples. For the base case, average values for all parameters shown in Figure 5 were used. A series of additional simulations were then run to see the effect of variation in individual parameter values on the base case. For each additional simulation, the base case was modified with either the 25th or the 75th percentile value of an input variable, while all other parameters were held at the values used for the base case. For example, the result labeled "Alk25" uses the 25th percentile for alkalinity (shown in Figure 5), and the result "Alk75" uses the 75th percentile for alkalinity. Sensitivity results for other parameters are labeled with a similar labeling scheme.

VI. PREDICTED ESTIMATE OF WQC

With the WER calculated in Section IV, site specific acute and chronic WQC can be calculated for the site. The site specific criteria are calculated as the state standards times the WER. For the receiving water downstream of the site, the average WER is 2.6, resulting in a site specific acute WQC of 614.1 µg/L and a site specific chronic WQC of 37.2 µg/L (Table 4).

Table 4. Summary of values for corresponding acute [a] and chronic [b] standards, WER, and resulting site specific standards in receiving water samples downstream of the plant. The Illinois acute and chronic standards for Nickel are based on hardness dependent equations. The average for samples collected in this study are based on the average measured hardness in samples collected for the BLM analysis. Also shown are the site-specific values based on a hardness of 359, which was assigned by the State of Illinois for this site.

Sample Date	Sample Location	Hardness	Nickel Acute[a] Standard	Nickel Chronic[b] Standard	Water Effect Ratio	Site Specific Acute Standard	Site Specific Chronic Standard
		mg/L as $CaCO_3$	µg/L	µg/L		µg/L	µg/L
8/26	RD at Rock Springs	357	241.7	14.7	2.6	628.5	38.1
9/9	RD at Rock Springs	360	243.5	14.8		633.0	38.4
8/26	RD at Lincoln	332	227.3	13.8	2.6	591.1	35.8
9/9	RD at Lincoln	341	232.5	14.1		604.6	36.6
	Average (this study)	**347.5**	**236.2**	**14.3**	**2.6**	**614.1**	**37.2**
	Site specific values using Illinois EPA-assigned critical hardness	**359**	**242.9**	**14.7**	**2.6**	**631.5**	**38.2**

Notes:

[a:] Nickel Acute Standard = exp[A+B*ln(H)] * 0.998 (where A=0.5173; B=0.846)

[b:] Nickel Chronic Standard = exp[A+B*ln(H)] * 0.997 (where A= -2.286; B=0.846)

VII. CONCLUSIONS

Water quality factors such as pH, alkalinity, ion content, and the presence of natural organic matter have been shown to affect metal toxicity. However, the WCQ for many metals consider only hardness, making them potentially over-protective or under-protective for many site waters. The BLM is a mechanistic framework suitable for a number of metals, including Nickel, which allows for the consideration of many additional water quality factors. The BLM has been adopted by US EPA in the most recently updated metals criteria (US EPA, 2007). For metals that do not yet have an approved WQC approach, the BLM can be used to calculate a WER adjustment to derive site specific acute and chronic criteria. Application of the Nickel BLM to calculate Nickel toxicity in samples taken from the Sangamon River downstream of the District's Main Plant compared to a reference water results in a calculated average WER of 2.6. This WER results in a site specific acute criterion of 614.1 µg/L and a site specific chronic criterion of 37.2 µg/L at a hardness equal to 347.5 mg/L. Utilizing the Illinois EPA-assigned hardness of 359 mg/L, the WER results in a corresponding acute criterion of 631.5 µg/L and a site specific chronic criterion of 38.2 µg/L.

VIII. REFERENCES

Carlson, A.R., W.A. Brungs, G.A. Chapman and D.J. Hansen. 1984. Guidelines for deriving numerical aquatic site specific water quality criteria by modifying national criteria. EPA-600/3-84-099, US EPA, Environmental Research Laboratory, Duluth, Minnesota, USA.

Di Toro, D.M., H.E. Allen, H.L. Bergman, J.S. Meyer, P .R. Paquin and R.C. Santore, 2001. A Biotic Ligand Model of the Acute Toxicity of Metals. I. Technical Basis, Environmental Toxicology and Chemistry. 20:2383-2396.

Erickson, R.J., D.A. Benoit, V.R. Mattson, H.P. Nelson, Jr. and E.N. Leonard. 1996. The effects of water chemistry on the toxicity of copper to fathead minnows. Environ. Toxicol. Chem. 15(2):181-193.

Keithly, J; J.A. Booker; D.K DeForest; B.K. Wu, and K.V. Brix. 2004. Acute and Chronic Toxicity of Nickel to a Cladoceran (Ceriodaphnia dubia) and an Amphipod (Hyalella azteca). Environmental Toxicology and Chemistry. 23(3):691-696

Santore, R.C., D.M. Di Toro and P.R. Paquin, H.E. Allen, and J.S. Meyer. 2001. "A Biotic Ligand Model of the Acute Toxicity of Metals. II. Application to Acute Copper Toxicity in Freshwater Fish and Daphnia," Environmental Toxicology and Chemistry. 20(10):2397-2402.

Stephan, C.E., D.I. Mount, D.J. Hansen, J.H. Gentile, G.A. Chapman and W.A. Brungs. 1985. Guidelines for deriving numerical national water quality criteria for the protection of aquatic organisms and their uses. PB85- 227049. National Technical Information Service, Springfield, VA.

US EPA. 1992. Interim guidance on interpretation and implementation of aquatic life criteria for metals. Health and Ecological Criteria Division, Office of Science and Technology, US EPA,Washington, District of Columbia, USA.

US EPA. 1994a. Water quality standards handbook. 2nd Edition. EPA-823-B94-005b. National Technical Information Service, Springfield, VA.

US EPA. 1994b. Interim Guidance on Determination and Use of Water-Effect Ratios for Metals. EPA-823-B-04-001. National Technical Information Service, Springfield, VA.

US EPA. 2007. Luis A. Cruz, Cindy Roberts, Mary Reiley, Robert Santore, Paul Paquin, Gary Chapman, Jennifer Mitchell, Charles Delos, Joseph Meyer, Rooni Mathew, and Tyler K. Linton. 2007. Aquatic Life Ambient Freshwater Quality Criteria - Copper. 2007 Revision. EPA-822-R-07-001.

WERF. 2003. Development of a Biotic Ligand Model for Nickel: Phase I. KB Wu, PR Paquin, V Navab, R Mathew, RC Santore, DM Di Toro. WERF 01-ECO-10T

Attachment B

OSU Aquatic Toxicology Laboratory
33972 Texas St. SW
Albany, Oregon 97321 USA
Office 541-737-2565
Lab 541-926-1254

Oregon State UNIVERSITY OSU

Title:	Water-Effect Ratio (WER) Testing of Acute Nickel Toxicity in Site Effluent Water and Laboratory Water to the Cladoceran, *Ceriodaphnia dubia*, under Static Test Conditions
Testing Facility:	Oregon State University Aquatic Toxicology Laboratory (OSU AquaTox) 33972 Texas Street SW Albany, OR 97321 USA
Study Sponsor:	Sanitary District of Decatur 501 S. Dipper Lane Decatur, Illinois 62522
Sponsor's Study Officer:	Timothy R. Kluge, Technical Director
Principal Investigator:	William Stubblefield, Ph.D.
Study Director:	Allison Cardwell

1.0 INTRODUCTION

1.1 Objective

To utilize the water-effect ratio (WER) procedure to determine the acute effects of nickel on the freshwater cladoceran, *Ceriodaphnia dubia*, in site effluent water and laboratory reconstituted water, under static test conditions.

1.2 Experimental Approach

C. dubia will be continuously exposed to differing concentrations of nickel in both site effluent water and laboratory reconstituted water during acute aqueous exposures.

1.3 Test Substance

The test substance will be in the form of nickel chloride hexahydrate ($NiCl_2$ x $6H_2O$; CAS # 7791-20-0).

2.0 BASIS AND TEST SYSTEM

2.1 Basis

This protocol is designed to comply with USEPA testing guidance (USEPA 2002) and WER procedures (USEPA 1994).

2.2 Test Species

1. Species: Cladoceran/Water Flea (*Ceriodaphnia dubia*).
2. Number: A total of 20 organisms will be tested for each treatment and control (four replicates per treatment).
3. *C. dubia* will start as less than 24 hr old neonates.
4. Source: *C. dubia* are cultured at Oregon State University's Aquatic Toxicology Lab (OSU AquaTox, Albany, OR).
5. Culture/Holding Water: For acclimation of organisms to the expected hardness of the site effluent water, *C. dubia* adults are maintained in a mass culture in very hard reconstituted water (nominal hardness, alkalinity, and pH of approximately 305 mg/L as $CaCO_3$, 225 mg/L as $CaCO_3$, and 8.5, respectively). In order to track reproduction of test organisms, at least two weeks prior to testing, organisms will be maintained individually in 30 mL plastic containers in an environmental chamber.
6. Feeding: No feeding will occur during the conduct of the test. Prior to the initiation of the test, < 24 hr old neonates will be grouped together and fed a suspension of Yeast/Trout Chow/Cereal leaves mixture (YTC) and algae suspension (*Pseudokirchneriella subcapitata*, 1:1) for at least 2 hours prior to the test. Organisms (which have been acclimated to very hard reconstituted water) for the site effluent testing will be acclimated to the site water for as long as possible prior to the test without compromising the time constraints of first use of the site water.
7. Procedure for identification: *C. dubia* have been verified to species by the original organism supplier.

2.3 Test Diet

The organisms will not be fed during the toxicity test.

3.0 EXPOSURE SYSTEM

3.1 Route of Administration

Method: Appropriate volumes of nickel stock will be mixed with both the site effluent water and the laboratory reconstituted water to achieve nominal concentrations. Following the spiking of nickel to the site effluent, the waters will be serially diluted and allowed to equilibrate for 2-4 hours prior to use. Following spiking of nickel to the laboratory reconstituted water, the waters will be serially diluted and allowed to equilibrate for 1-3 hours.

Equipment: The laboratory reconstituted water will be prepared with reagent grade salts (see section 1.3) and will be weighed/apportioned using an electronic micro-balance and micro-pipettes.

Frequency: This is a static test. No water renewal will occur during the conduct of the test.

3.2 Dilution Water

Dilution water for the laboratory water test will be a very hard reconstituted laboratory water made from deionized water amended with the appropriate reagent grade salts ($CaSO_4 \cdot 2H_2O$, $MgSO_4$, KCl, and $NaHCO_3$) to achieve a nominal hardness, alkalinity, and pH of approximately 315 mg/L as $CaCO_3$, 225 mg/L as $CaCO_3$, and 8.0, respectively. Reconstituted water will be prepared as detailed in standard USEPA methods (USEPA 2002) with a Ca to Mg (Ca:Mg) ratio of 0.7.

The site effluent water will not be diluted.

3.3 Test Temperature

Test temperature will be 25 ± 2 °C. Testing will be conducted in a temperature-controlled environmental chamber.

3.4 Test Chamber

Test containers will be 30-mL plastic Soufflé cups containing 25-mL of test solution. Containers will be covered with Plexiglas to prevent contamination.

3.5 Photoperiod

Lighting for the entire test duration will be a photoperiod of 16-hours light and 8-hours dark, provided by cool-white or daylight illumination.

3.6 Dissolved Oxygen Concentrations

Dissolved oxygen concentrations will be maintained at ≥ 60 percent of saturation.

4.0 TEST DESIGN

4.1 Test Concentrations/Dosages

For each test (site effluent and laboratory water), five test treatments and a control will be tested using a 0.7 dilution scheme. The nominal test concentrations will be estimated based upon expected acute toxicity of nickel based upon the hardness and pH of the waters and historical data. Nominal test concentrations will be described in the raw data packet. A concurrent moderately hard reconstituted control water (USEPA 2002; without nickel) will also be tested.

4.2 Number of Test Organisms

For each test (site effluent and laboratory water), a total of 20 organisms will be tested in four replicates for each treatment and control. A concurrent moderately hard reconstituted water control (without nickel) will be tested in the same conditions as the site effluent and laboratory water. Five *C. dubia* will be randomly partitioned into each test vessel at the start of the test.

4.3 Bias Control

To control bias, test chambers will be numbered according to a 4 X 6 randomization sheet and placed in the environmental chamber.

4.4 Test Initiation

Following preparation of each concentration, solutions will be allocated to each replicate. Organisms will then be randomly allocated into each replicate until 5 organisms are in each chamber.

4.5 Chemical and Physical Monitoring

At a minimum, the following measurements will be made according to the methods laid out in OSU AquaTox SOPs:

1. Hardness, alkalinity, dissolved oxygen, temperature, conductivity, total ammonia, total residual chlorine, and pH will be measured in the site effluent water and the laboratory reconstituted water at test initiation. Hardness and pH of the control, one middle concentration and the highest concentration, will also be measured at the 48 hour renewal time point (of both new renewal waters and old waters) and at test termination.

2. A sample of the site effluent water and the laboratory reconstituted water will be collected for characterization of calcium, magnesium, sodium, potassium, chloride, sulfate, and dissolved organic carbon (DOC) and measured at an outside commercial laboratory.

3. Dissolved oxygen, temperature, conductivity, and pH will be measured daily in each treatment.

4.6 Biological Monitoring

Observations of live and dead organisms will be recorded daily. Dead organisms will be removed immediately following observation.

4.7 Analytical Chemistry

Samples for nickel analysis will be collected from each treatment according to the following schedule: On Day 0 (initiation), samples for total recoverable (unfiltered and acidified with concentrated nitric acid to a pH < 2) and dissolved (filtered through 0.45 µm-porosity filter prior to acidification) will be collected separately into a 15 ml polypropylene conical tube from each treatment. Samples for analysis of total and dissolved nickel will also be collected from old test waters (from a composite of the four replicates for each treatment) at test termination. Filters (0.45 µm-porosity) used for dissolved metal collections will be flushed with 5 ml of sample prior to sample collection. Total recoverable and dissolved nickel samples will be analyzed via Inductively Coupled Plasma Optical Emission Spectrometry or Mass Spectrometry (ICP-OES/MS).

4.8 Test Duration

The test duration will be 48 hours ± 1 hour.

4.9 Quality Criteria

- The test will not be considered valid if control mortality exceeds 10%.
- The dissolved oxygen concentration must be > 60 percent saturation.
- There must be evidence that the temperature, dissolved oxygen, and concentration of the test substance being tested have been satisfactorily maintained, based on time-weighted averages, over the test period.

5.0 DATA ANALYSIS

Statistical analysis (hypothesis testing) of the test data will be conducted using a computer program. A statistical test (as determined by the USEPA Decision Tree in acute toxicity test guidance [USEPA, 2002]) will be used to test for significant differences in the survival among test treatments and controls. EPA methodology (2002) dictates the flowchart for determination of the LC50 for multi-effluent concentration acute toxicity tests and this flowchart coincides with the statistical methodology described in the WER guidance (1994).The no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) will be calculated on the basis of survival ($p < 0.05$). In addition, a median lethal concentration (LC50) will be calculated along with the determination of outliers and the need for data transformation (i.e., arc sine, square root, logarithmic, etc.).

The experimentally determined WER will typically be a ratio of endpoints determined at two different hardnesses and will thus include contributions from a variety of differences between the two waters, including hardness. The WER will be calculated as discussed in the guidance document (USEPA 1994). The use of the USEPA very hard reconstituted laboratory water as a reference water is a reasonably close match to the site effluent hardness. The reference water

LC50 may be further adjusted to match the site water hardness using the hardness slope for the Ni standard.

6.0 TEST REPORT

The report will be a typed document describing the results of the test and will be signed by the Principal Investigator and Study Director. The report will include, but not be limited to, the following:

- Name and address of the test facility;
- Dates of test initiation, completion, and/or termination;
- Objectives of the study as stated in the test protocol, including any changes from the protocol;
- Statistical methods used in data analysis;
- Identification of the test substances (by name, CAS number, or code number) and description of substance purity, strength, composition, stability, solubility, and/or other appropriate characteristics documented by the Study Sponsor (location of documentation shall be specified);
- A description of the methods used during testing;
- A description of the test system used including, where applicable, algal density or biomass, source of supply, species, strain, sub-strain, age, and procedure for identification;
- A description of the exposure concentrations, dosing regimen, route of administration, and duration of exposure;
- A description of all circumstances that may have affected the quality and/or integrity of the data;
- The name of the Principal Investigator and Study Director and the names of other scientists, professionals, or supervisory personnel (e.g. task manager, senior biomonitoring technician) involved in the study;
- A description of the methods of data analysis; a summary and analysis of the data, and a statement of the conclusions drawn from the analysis;
- Signature and date of the Study Director and/or other professionals involved in the study as required by the testing facility or Sponsor;
- The location(s) where all specimens, raw data, and final report are to be stored;
- A statement of Quality Assurance

7.0 RECORD RETENTION

All records will be maintained and archived in the OSU AquaTox archives in accordance with OSU AquaTox SOP 5403.

8.0 PROTOCOL AMENDMENTS AND DEVIATIONS

All changes (i.e., amendments, deviations, and final report revisions) of the approved protocol, plus the reasons for the changes, must be documented in writing. The changes will be signed and dated by the Study Director and maintained with the protocol.

OSU Aquatic Toxicology Laboratory
33972 Texas St. SW
Albany, Oregon 97321 USA
Office 541-737-2565
Lab 541-926-1254

Oregon State UNIVERSITY OSU

Title:	Water-Effect Ratio (WER) Testing of Acute Nickel Toxicity in Site Effluent Water and Laboratory Water to the Fathead Minnow, *Pimephales promelas*, under Static-Renewal Test Conditions
Testing Facility:	Oregon State University Aquatic Toxicology Laboratory (OSU AquaTox) 33972 Texas Street SW Albany, OR 97321 USA
Study Sponsor:	Sanitary District of Decatur 501 S. Dipper Lane Decatur, Illinois 62522
Sponsor's Study Officer:	Timothy R. Kluge, Technical Director
Principal Investigator:	William Stubblefield, Ph.D.
Study Director:	Allison Cardwell

1.0 INTRODUCTION

1.1 Objective

To utilize the water-effect ratio (WER) procedure to determine the acute effects of nickel on the fathead minnow, *Pimephales promelas*, in site effluent water and laboratory reconstituted water, under static-renewal test conditions.

1.2 Experimental Approach

P. promelas will be continuously exposed to differing concentrations of nickel in both site effluent water and laboratory reconstituted water during acute aqueous exposures.

1.3 Test Substance

The test substance will be in the form of nickel chloride hexahydrate ($NiCl_2$ x $6H_2O$; CAS # 7791-20-0).

2.0 BASIS AND TEST SYSTEM

2.1 Basis

This protocol is designed to comply with USEPA testing guidance (USEPA 2002) and WER procedures (USEPA 1994).

2.2 Test Species

1. Species: Fathead Minnow (*Pimephales promelas*).
2. Number: Each test will consist of a total of 40 organisms per treatment, with each treatment containing four replicates. Ten larval fish will be partitioned into each vessel at the start of the test.
3. Age: Larval fish, *P. promelas* (~7-14 days old), at start of test.
4. Source: *P. promelas* will be obtained from in-house cultures located at OSU AquaTox.
5. Holding Conditions: *P. promelas* adults are maintained in brood tanks using a continuous flow-through system with natural well water that is saturated with dissolved oxygen and has a nominal hardness, alkalinity, and pH of approximately 100 mg/L as $CaCO_3$, 100 mg/L as $CaCO_3$, and 7.8, respectively.
6. Larval fish will be held in holding tanks with feeding and water renewal prior to use in the toxicity test. Fish will be acclimated to the very high hardness conditions by daily water renewals, increasing hardness by approximately 50 mg/L as $CaCO_3$, daily. Fish will be approximately 7-14 days old at test initiation. Organisms (which have been acclimated to very hard reconstituted water) for the site effluent testing will be acclimated to the site water for as long as possible prior to the test without compromising the time constraints of first use of the site water.
7. Feeding: At the 48-hour time point in testing, 0.2 mL brine shrimp nauplii will be fed to each test chamber. Feeding will be allowed for a minimum of 2 hours, prior to solution renewal.

8. Procedure for identification: *P. promelas* have been verified to species by the original organism supplier.

2.3 Test Diet

Brine shrimp cysts (Brine Shrimp Direct, Ogden UT, USA) are hatched in the laboratory and are typically < 30 hours old (*Artemia* nauplii) when fed to the test organisms. A sample of newly-hatched *Artemia* nauplii are chemically analyzed on an annual basis for total metals, organochlorine pesticides, and PCBs as per OSU AquaTox's Standard Operation Procedures (SOPs).

3.0 EXPOSURE SYSTEM

3.1 Route of Administration

Method: Appropriate volumes of nickel stock will be mixed with both the site effluent water and the laboratory reconstituted water to achieve nominal concentrations. Following the spiking of nickel to the site effluent, the waters will be serially diluted and allowed to equilibrate for 2-4 hours prior to use. Following spiking of nickel to the laboratory reconstituted water, the waters will be serially diluted and allowed to equilibrate for 1-3 hours.

Equipment: The laboratory reconstituted water will be prepared with reagent grade salts (see section 1.3) and will be weighed/apportioned using an electronic micro-balance and micro-pipettes.

Frequency: An 80% renewal of control and treatment solutions will occur at 48 hours by siphoning out 80% of the old water and waste and pouring freshly prepared test solutions (following the aging periods stated in the method above), as appropriate, back into the chambers.

3.2 Dilution Water

Dilution water for the laboratory water test will be a very hard reconstituted laboratory water made from deionized water amended with the appropriate reagent grade salts ($CaSO_4 \cdot 2H_2O$, $MgSO_4$, KCl, and $NaHCO_3$) to achieve a nominal hardness, alkalinity, and pH of approximately 315 mg/L as $CaCO_3$, 225 mg/L as $CaCO_3$, and 8.0, respectively. Reconstituted water will be prepared as detailed in standard USEPA methods (USEPA 2002) with a Ca to Mg (Ca:Mg) ratio of 0.7.

The site effluent water will not be diluted.

3.3 Test Temperature

Test temperature will be 25 ± 2 °C. Testing will be conducted in a temperature-controlled environmental chamber.

3.4 Test Chamber

Test containers will be 250-mL glass beakers containing 200-mL of test solution. Containers will be covered with Plexiglas to prevent contamination.

3.5 Photoperiod

Lighting for the entire test duration will be a photoperiod of 16-hours light and 8-hours dark, provided by cool-white or daylight illumination.

3.6 Dissolved Oxygen Concentrations

Dissolved oxygen concentrations will be maintained at ≥ 60 percent of saturation.

4.0 TEST DESIGN

4.1 Test Concentrations/Dosages

For each test (site effluent and laboratory water), five test treatments and a control will be tested using a 0.7 dilution scheme. The nominal test concentrations will be estimated based upon expected acute toxicity of nickel based upon the hardness and pH of the waters and historical data. Nominal test concentrations will be described in the raw data packet. A concurrent moderately hard reconstituted control water (USEPA 2002; without nickel) will also be tested.

4.2 Number of Test Organisms

For each test (site effluent and laboratory water), a total of 40 organisms will be tested in four replicates for each treatment and control. A concurrent moderately hard reconstituted water control (without nickel) will be tested in the same conditions as the site effluent and laboratory water. Ten larval fish will be randomly partitioned into each test vessel at the start of the test.

4.3 Bias Control

To control bias, test chambers will be numbered according to a 4 X 7 randomization sheet and placed in the environmental chamber.

4.4 Test Initiation

Following preparation of each concentration, solutions will be allocated to each replicate. Organisms will then be randomly allocated into each replicate until 10 organisms are in each chamber.

4.5 Chemical and Physical Monitoring

At a minimum, the following measurements will be made according to the methods laid out in OSU AquaTox SOPs:

1. Hardness, alkalinity, dissolved oxygen, temperature, conductivity, total ammonia, total residual chlorine, and pH will be measured in the site effluent water and the

laboratory reconstituted water at test initiation. Hardness and pH of the control, one middle concentration and the highest concentration, will also be measured at the 48 hour renewal time point (of both new renewal waters and old waters) and at test termination.

2. A sample of the site effluent water and the laboratory reconstituted water will be collected for characterization of calcium, magnesium, sodium, potassium, chloride, sulfate, and dissolved organic carbon (DOC) and measured at an outside commercial laboratory.

3. Dissolved oxygen, temperature, conductivity, and pH will be measured daily in each treatment.

4.6 Biological Monitoring

Observations of live and dead organisms will be recorded daily. Dead organisms will be removed immediately following observation.

4.7 Analytical Chemistry

Samples for nickel analysis will be collected from each treatment according to the following schedule: On Day 0 (initiation), samples for total recoverable (unfiltered and acidified with concentrated nitric acid to a pH < 2) and dissolved (filtered through 0.45 µm-porosity filter prior to acidification) will be collected separately into a 15 ml polypropylene conical tube from each treatment. Samples for analysis of total and dissolved nickel will also be collected on Day 2 from old test waters (from a composite of the four replicates for each treatment) and freshly prepared renewal waters. Total and dissolved metals samples will also be taken at test termination. Filters (0.45 µm-porosity) used for dissolved metal collections will be flushed with 5 ml of sample prior to sample collection. Total recoverable and dissolved nickel samples will be analyzed via Inductively Coupled Plasma Optical Emission Spectrometry or Mass Spectrometry (ICP-OES/MS).

4.8 Test Duration

The test duration will be 96 hours ± 1 hour.

4.9 Quality Criteria

- The test will not be considered valid if control mortality exceeds 10%.
- The dissolved oxygen concentration must be > 60 percent saturation.
- There must be evidence that the temperature, dissolved oxygen, and concentration of the test substance being tested have been satisfactorily maintained, based on time-weighted averages, over the test period.

5.0 DATA ANALYSIS

Statistical analysis (hypothesis testing) of the test data will be conducted using a computer program. A statistical test (as determined by the USEPA Decision Tree in acute toxicity test guidance [USEPA, 2002]) will be used to test for significant differences in the survival among

test treatments and controls. EPA methodology (2002) dictates the flowchart for determination of the LC50 for multi-effluent concentration acute toxicity tests and this flowchart coincides with the statistical methodology described in the WER guidance (1994).The no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) will be calculated on the basis of survival ($p < 0.05$). In addition, a median lethal concentration (LC50) will be calculated along with the determination of outliers and the need for data transformation (i.e., arc sine, square root, logarithmic, etc.).

The experimentally determined WER will typically be a ratio of endpoints determined at two different hardnesses and will thus include contributions from a variety of differences between the two waters, including hardness. The WER will be calculated as discussed in the guidance document (USEPA 1994). The use of the USEPA very hard reconstituted laboratory water as a reference water is a reasonably close match to the site effluent hardness. The reference water LC50 may be further adjusted to match the site water hardness using the hardness slope for the Ni standard.

6.0 TEST REPORT

The report will be a typed document describing the results of the test and will be signed by the Principal Investigator and Study Director. The report will include, but not be limited to, the following:

- Name and address of the test facility;
- Dates of test initiation, completion, and/or termination;
- Objectives of the study as stated in the test protocol, including any changes from the protocol;
- Statistical methods used in data analysis;
- Identification of the test substances (by name, CAS number, or code number) and description of substance purity, strength, composition, stability, solubility, and/or other appropriate characteristics documented by the Study Sponsor (location of documentation shall be specified);
- A description of the methods used during testing;
- A description of the test system used including, where applicable, algal density or biomass, source of supply, species, strain, sub-strain, age, and procedure for identification;
- A description of the exposure concentrations, dosing regimen, route of administration, and duration of exposure;
- A description of all circumstances that may have affected the quality and/or integrity of the data;
- The name of the Principal Investigator and Study Director and the names of other scientists, professionals, or supervisory personnel (e.g. task manager, senior biomonitoring technician) involved in the study;
- A description of the methods of data analysis; a summary and analysis of the data, and a statement of the conclusions drawn from the analysis;
- Signature and date of the Study Director and/or other professionals involved in the study as required by the testing facility or Sponsor;
- The location(s) where all specimens, raw data, and final report are to be stored;
- A statement of Quality Assurance

7.0 RECORD RETENTION

All records will be maintained and archived in the OSU AquaTox archives in accordance with OSU AquaTox SOP 5403.

8.0 PROTOCOL AMENDMENTS AND DEVIATIONS

All changes (i.e., amendments, deviations, and final report revisions) of the approved protocol, plus the reasons for the changes, must be documented in writing. The changes will be signed and dated by the Study Director and maintained with the protocol.

9.0 LITERATURE CITED

USEPA. 1994. Interim Guidance on Determination and Use of Water-Effect Ratios for Metals. Office of Water. Washington, D.C. EPA-823-B-94-001.

USEPA. 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fifth Edition. EPA-821-R-02-012.

10.0 SPONSOR AND STUDY DIRECTOR APPROVAL

Sponsor :

Print Name: _____
Signature: _____
Title: _____
Date: _____

Principal Investigator:

Print Name: William Stubblefield
Signature: [signature]
Title: Professor
Date: 18 APRIL 2014

Study Director:

Print Name: Allison Cardwell
Signature: Allison Cardwell
Title: Faculty Research Assistant
Date: 18 April 2014

Exhibit 25

Comment 1. Thank you for clarifying.

Comment 2. It does not appear as though the calcium or magnesium values included in Table 3 of the "Estimate of the BLM Adjustment to the Nickel Criterion for the Sanitary District of Decatur, Illinois" document contained within the "WER study plan with attachments rev 4_23_14.docx" file provided on April 23, 2014 have changed since the April 16, 2013 version of the "Estimate" report. If there is a newer version of the report that includes these revisions, please feel free to forward this to EPA.

In addition, EPA looks forward to receiving the information necessary to verify the performance of the nickel BLM.

Comment 3. EPA understands that the proposal is to modify IL's criterion and not the national recommended criterion.

EPA acknowledges that IL's acute nickel criterion to protect aquatic life at a hardness of 50 mg/L is 45.9 µg/L and that Keithly *et al.*'s study identified a nickel LC50 value of 81 µg/L at 50 mg/L of hardness. However, during development of its nickel criterion, IL identified a *Ceriodaphnia* genus mean acute value (GMAV) (normalized to a hardness of 50 mg/L) of 30.16 µg/L. This GMAV was derived using data generated by Schubauer-Berigan *et al*., who identified *C. dubia* LC50 values of 32.6 µg/L (at pH = 7.1) and 3.03 µg/L (at pH = 8.6) (after normalizing to a hardness of 50 mg/L). Therefore, based on other data in the scientific literature, there appears to be uncertainty around the concentration of nickel necessary to generate toxic effects in *C. dubia* at a hardness of 50 mg/L.

If the Sanitary District of Decatur (SDD) wishes to pursue the use of *C. dubia* as a test organism, it may be possible to perform initial range-finding tests in very hard water and site water to determine how the LC50 values compare to IL's hardness-adjusted CMC. If these tests demonstrate that *C. dubia*'s LC50 value is less than or equal to the CMC, then the use of an alternative test organism (such as *Daphnia* sp.) would be consistent with EPA's guidance.

Comment 4. Thank you for clarifying and updating the test protocol.

Comment 5. IL's chronic nickel criterion was developed using toxicological data, not an acute-chronic ratio.

Given that SDD proposes to modify a chronic criterion, a chronic WER seems as though it would be most directly applicable.

Comment 6. Thank you for addressing this comment.

Comment 7. Can you please provide additional information on site water hardness (vs. proposed test water)? While EPA agrees that the waters are similar, additional analysis of the difference in hardness values and any effects on WER calculations should be explored. In particular, EPA recommends considering EPA's guidance document entitled "Use of the WER Procedure with Hardness Equations" for any further adjustments to hardness.

Comment 8. Thank you for addressing this comment.

Comment 9. Thank you for addressing this comment.

Comment 10. Calcium precipitation has been reported in testing using EPA hard water. Therefore, EPA recommends that hardness (as well as pH) are monitored at the beginning and end of testing. It appears that SDD's sampling protocol will address EPA's major concern, although researchers should note when and if precipitation occurs. If precipitation is common and significant, it may be advantageous to conduct sampling of ions at the end of the test in a subset of test waters.

In addition, EPA notes that static test conditions are susceptible to low dissolved oxygen (DO) conditions and that SDD proposes to monitor DO (as well as temperature, conductivity, and pH) daily over the course of the procedure. How will these measurements be carried out? Will probes be used to measure DO and other physiochemical variables in experimental chambers? If so, what steps will be taken to prevent the introduction of probes from inadvertently influencing experimental results (*e.g.*, via material transfer from one test chamber to another)? Or, will chemistry controls (*i.e.*, treatment waters in chambers without organisms) be used to measure physiochemical variables, including DO?

Comment 11. It is unclear which document "the newest EPA acute version (2002)" is referencing. Will SDD please provide the full citation and/or a link to the document?

Comment 12. It is not clear whether the age of fathead minnows used in the proposed test will be similar to the ages of the organisms used to derive IL's criteria and/or the nickel BLM. What steps will be taken to prevent age-related differences in nickel sensitivity from influencing the WER?

Exhibit 26

Draft Manuscript

Running head: A review of water quality factors that affect nickel bioavailability to aquatic organisms: Refinement of the Biotic Ligand Model for Nickel in acute and chronic exposures

Corresponding author: Robert Santore

Windward Environmental, LLC, East Syracuse, NY 13057 USA

E-mail: RobertS@windwardenv.com

Telephone: 206-812-5450

Number of words: 9776

Robert C. Santore*, Kelly Croteau, Adam C. Ryan

Windward Environmental, LLC, 5848 Marlow Dr., East Syracuse, NY 13057 USA

* RobertS@windwardenv.com

Introduction

Nickel toxicity can be affected by a wide variety of chemical parameters, such as pH, hardness, and the presence of natural organic matter and these factors have previously been considered in the development of a Biotic Ligand Model (BLM) for Ni (Wu et al., 2003). Regulatory approaches for Ni, however, typically consider only hardness (US EPA, 1980). In order to consider a wide variety of other factors identified, including DOC, pH, and alkalinity, the Ni BLM software was developed as part of a research project for WERF (Wu et al., 2003). Since the completion of the WERF study a lot of new research has been published to investigate water quality factors that affect Ni bioavailability, and these works greatly expand the number of organisms for which bioavailability data has been generated, the range of parameter testing, and includes chronic exposures. Given the availability of these new studies, and continued interest in using the Ni BLM to assess Ni bioavailability in regulatory and risk assessment settings, this review intends to test whether a bioavailability approach based on the Ni BLM can effectively predict Ni toxicity to aquatic organisms in a wide range of conditions.

- Objectives for this work were to:
    - summarize the water quality factors that have been shown to affect nickel bioavailability and toxicity for fish and invertebrates in acute and chronic exposures,
    - evaluate the consistency of observed effects and determine if a single model can be used across different taxonomic groups, different exposure durations, and different toxicological endpoints
    - revise the nickel BLM as needed to improve the degree to which it is predictive for nickel bioavailability in a wide range of conditions in acute and chronic exposures to fish and invertebrate organisms

Model Description

The Ni BLM software was originally developed as part of a research project for WERF (Wu et al., 2003) following the development of BLM versions for Cu (Di Toro et al., 2001; Santore et al., 2001) and Zn (Santore et al., 2002). The Ni BLM shares the same overall conceptual model used for these other metals in that bioavailability is described as the interactions between factors that affect metal speciation and factors that affect metal accumulation on biological membranes (Figure 1). This shares conceptual elements with the Gill Surface Interaction Model proposed by Pagenkopf (1983) as well as the free ion activity model (Morel, 1983; Morel and Hering, 1993; Campbell, 1995). The accumulation of metals on the biotic ligand is the pathway by which toxic effects occur in organisms, often interfering with other necessary processes. The chemical speciation calculations are performed with CHESS (Santore and Driscoll, 1995), a framework that solves the system of equations associated with chemical equilibria and the charge balance. The Windermere Humic Aqueous Model (WHAM Version 1.0, Model V, Tipping 1994) is incorporated into the CHESS framework within the BLM in order to model the interactions of metals with organic matter. The use of WHAM is advantageous since it has been calibrated with a large dataset consisting of many sources of organic matter, over a wide range of chemical conditions, and for several metals including nickel.

Data Review

A literature review was performed to identify papers that reported Ni toxicity to aquatic fish and invertebrates in exposures that included a range of water chemistry. The review included studies that covered a wide variety of organisms, reported endpoints, exposure durations, and toxic effects. The studies and reported toxicity data identified in this review are summarized in Table 1. When multiple endpoints were reported in a study, the more robust endpoint was preferentially used for model comparison (e.g., EC50s were preferred over EC20, and EC20 were preferred over EC10s)

Studies selected for this review focused on variation in chemistry in synthetic and natural samples. Studies with synthetic samples used a pure water source with salt additions to design a series of conditions such that the exposure chemistry varied in a systematic way (e.g., variation in hardness) and were used in the calibration phase of model refinement. Studies with natural water samples tended to select sampling sites that provided diverse water chemical characteristics where multiple chemical parameters may co-vary from sample to sample. Studies that quantified Ni toxicity on natural water samples were used for validation of the calibrated model.

For all studies considered in this review, we required that important chemical parameters required by the BLM were measured. BLM parameters include pH, dissolved organic carbon (DOC), alkalinity, calcium, magnesium, sodium, potassium, sulfate, and chloride. Measurements for all parameters were preferred, but estimates for some parameters were acceptable if we felt that enough information was provided, or if the missing parameter was relatively unimportant. For example, if hardness was reported but little else, the estimates for the major ions were based on ion ratios calculated from another source of chemistry data with the same water body. Concentrations of DOC were only estimated in synthetic waters for which values near zero were expected. DOC was estimated as 0.3 mgC/L for acute tests and slightly higher at 0.5 mgC/L for chronic tests since feeding the organisms in the tests would contribute extra DOC. Alkalinity was estimated when necessary from pH by assuming equilibrium with atmospheric CO_2(g), such as:

$$[H_2CO_3^*] = 10^{-pCO_2} * K_H$$

$$[HCO_3^-] = \frac{K_1 * [H_2CO_3^*]}{[H^+]}$$

$$[CO_3^{2-}] = \frac{K_1 * [HCO_3^-]}{[H^+]}$$

$$Alkalinity\ (mg/L\ CaCO_3) = ([HCO_3^-] + 2 * [CO_3^{2-}] + [OH^-] - [H^+]) * \frac{100086\ mg\ CaCO_3}{2\ eq}$$

where $pCO_2 = 3.5$

$K_H = 10^{-1.5}$

$K_1 = 10^{-6.352}$

$K_2 = 10^{-10.329}$

Although the concentration of CO_2 indoors can sometimes be higher than what was used in this estimation, a validation of the alkalinity estimates compared to reported alkalinity showed that a pCO_2 of 3.5 gave an adequate prediction, and a lower value did not greatly change the estimation, nor improve

the fit to measured data. All chemical inputs used in this analysis were reported in the Supplemental information (see appendix), where estimated data are identified with a bold and italic font.

Estimation method for cation and anion concentrations

Data were available for hardness, pH, and alkalinity whenever the major ions required for the BLM were not reported. Average ion ratios were used to estimate the full chemistry for each sample. The average ratios of Ca^{2+} to Mg^{2+}, Na^{+}, and K^{+}; and SO_4^{2-} to Cl^{-} were calculated across available data found in various literature sources (see Table 4). For this calculation, the hardness was assumed to be equivalent to Ca + Mg, so that the Ca concentration for a given sample (i) was estimated as:

$$[Ca^{2+}]_i = \frac{Hardness_i}{1 + \frac{1}{Ca:Mg}}$$

Estimates for the other cations (Mg^{2+}, Na^{+}, and K^{+}) were calculated by dividing the Ca ion concentration by the respective ratios, such as:

$$[Ion]_i = \frac{[Ca^{2+}]_i}{Ca:Ion}$$

For anions, the concentrations of bicarbonate and carbonate were estimated from pH and reported alkalinity. The remaining anions were determined to satisfy an electroneutrality condition (i.e., the sum of the equivalent charges for cations and anions must be equal). Therefore the total concentration of SO_4^{2-} and Cl^{-} was determined for each month as the deficit of a charge balance with the cations, pH, and alkalinity.

$$[SO_4^{2-} + Cl^{-}] = 2[Ca^{2+}] + 2[Mg^{2+}] + [Na^{+}] + [K^{+}] + [H^{+}] - [HCO_3^{-}] - 2[CO_3^{2-}] - [OH^{-}]$$

where $[HCO_3^{-}] = \frac{[Alk] - [OH^{-}] + [H^{+}]}{1 + \frac{2 * K_2}{[H^{+}]}}$

$$[CO_3^{2-}] = K_2 * \frac{[HCO_3^{-}]}{[H^{+}]}$$

$$[H^{+}] = 10^{-pH}$$

$$[OH^{-}] = 10^{-(14 - pH)}$$

$K_2 = 10^{-10.329}$

and *Alk* = alkalinity in equivalents / L = 2 x 10^{-5} x alkalinity (as mg $CaCO_3$ / L). The concentrations of each anion were calculated using this deficit and the SO_4: Cl ion ratio:

$$[SO_4^{2-}] = \frac{[SO_4^{2-} + Cl^{-}]}{2 + \frac{1}{SO_4:Cl}}$$

$$[Cl^{-}] = \frac{[SO_4^{2-}]}{SO_4:Cl}$$

Summary of Ni bioavailability literature used in this analysis

Based on these search criteria, a number of studies were selected for model calibration and evaluation. For acute exposures, fourteen studies were selected that included toxicity data for fifteen species including fourteen invertebrates and one fish (Table 1). Chapman et al (1980) measured Ni toxicity to *D. magna* in acute exposures that varied hardness and alkalinity concentrations. Deleebeeck et al (2007a) reported toxicity data for acute exposures in synthetic water with 10 cladoceran species originating from various soft (S) or hard (H) surface waters. Individual species originating from hard surface waters were tested in moderately hard and hard water, while those originating from soft water were tested in soft and moderately hard water. Deleebeeck et al (2008) measured Ni toxicity to *D. magna* in acute exposures with synthetic and natural waters including five series of toxicity tests in synthetic waters which varied Ca, Mg, Na, pH (with $NaHCO_3$ controlling pH), and a second series with pH (with MOPS + NaOH/HCl controlling pH). Doig and Liber (2006) measured acute Ni toxicity to *H. azteca* in synthetic water containing varying amounts of DOC from multiple sources, in whole peat, peat hydrophilic DOC, humics, and fulvics forms. Hoang et al (2004) measured Ni toxicity in acute exposures using either <1 day or 28 day old fathead minnow (*P. promelas*). Individual toxicity tests in the Hoang et al (2004) study included variation in alkalinity, hardness, pH, or DOC concentrations. In addition to the data reported in 2004, additional data from a subsequent unpublished experiment are included (see Supplemental Table 5). Keithly et al (2004) performed acute exposures to measure Ni toxicity on *C.dubia* in synthetic water with variable hardness. Kozlova et al (2009) measured Ni toxicity to *D. pulex* in acute exposures with synthetic water in eight toxicity test series that varied concentrations in Ca, K, Mg, Na, DOC from the Nordic Reservoir, DOC from the Suwannee River, pH, and pH controlled by MOPS. Lind et al (1978) measured Ni toxicity in acute exposures using *D. pulicaria* and *P. promelas* in six different natural waters. Meyer et al (1999) performed acute exposures on sub-adult *P. promelas* to measure Ni toxicity at varying Ca concentrations. Pickering (1974) performed duplicate toxicity tests on 100-day-old *P. promelas* in hard and soft waters to measure acute Ni toxicity. Pyle et al (2002) measured acute Ni toxicity on larval *P. promelas* at different hardness concentrations and different pH values. Schubauer-Berigan et al (1993) measured acute Ni toxicity to *C. dubia* and *P. promelas* with variable pH.

For chronic exposures, five studies were selected with measurements from 13 species including 11 invertebrates, one fish, and one plant (Table 1). Deleebeeck et al (2007a) reported reproduction and survival chronic Ni toxicity endpoints for 9 of the 10 cladocerans (*B. coregoni* excluded) for which they also reported acute toxicity (see above). Some cladocerans showed no reproduction during the 16-21 day exposure period, and so only had a survival endpoint reported. Deleebeeck et al (2007b) measured chronic Ni toxicity on *O. mykiss* in synthetic and natural waters. Toxicity tests in synthetic waters were performed in 3 series, varying Ca, Mg, and pH concentrations. Keithly et al (2004) tested chronic Ni toxicity to *C. dubia* at varying hardness concentrations, with endpoints of reproduction and survival. In an unpublished study, Parametrix measured chronic Ni toxicity to *C. dubia* in natural and synthetic waters. Toxicity test series were performed in synthetic waters that varied alkalinity and hardness, and pH. Toxicity tests were also performed in three natural water samples, in the raw water, filtered water, and (for two of the waters) in synthetic water made to match the natural chemistry with and without DOC from that source. Schlekat et al (2010) tested chronic Ni toxicity in four natural waters, one of which was tested with and without a pH adjustment (the objective being to get a wide range of water chemistry). Toxicity tests were done on three invertebrates (*B. calyciflorus*, *C. tentans*, and *L. stagnalis*) and one plant (*L. minor*) for various endpoints.

Bioavailability Effects & Model Calibration

In the original calibration of the Ni BLM, the log K values for Ni binding to biotic ligand sites were based on measurements of Ni accumulation on the gills of *P. promelas* reported by Meyer et al., 1999. Application of the Ni BLM for this review included refinement of model parameters in recognition of the

large amount of new data that have become available since the original Ni model was developed. Refinement of model parameters was performed as much as possible by considering a single parameter at a time, and comparing goodness of fit of the overall model with data that relates only to model responses to that single parameter. The parameters associated with specific water quality factors will be discussed one at a time in the sections that follow. After considering the single-parameter bioavailability experiments, the analysis was then expanded to include all data simultaneously to evaluate overall model performance in synthetic and natural waters. The ultimate goal of the model evaluation is to determine if observed Ni bioavailability factors are consistent with the conceptual model that uses chemical speciation and organism interactions to predict bioavailability, and to determine the extent to which a single set of BLM parameters could be used for all organisms in all conditions. The use of a single set of parameters in the BLM can simplify the use of model in regulatory contexts, and provides support for the interpretation of factors that affect Ni bioavailability as universal, mechanistically based processes that can be applied in a consistent way to all aquatic organisms in acute and chronic exposures. Figure 2A demonstrates this concept for the hardness effect, and Figure 2B for calcium. In these figures, the effect concentrations are normalized to the SMEA to account for the different sensitivities of different organisms. As can be seen, the effect concentrations fall closely on a single line with a significant slope, giving evidence not only that a single parameter can estimate the bioavailability effects for many organisms, but also that there is a notable hardness effect.

The goodness of fit measure used to select the calibrated Log K value was the coefficient of determination or R-squared on the logs of ECx values ($logR^2$). The $logR^2$ can be calculated as:

$$logR^2 = 1 - \frac{\sum_{i=1}^{N}(\log_{10} y_i - \log_{10} \hat{y}_i)^2}{\sum_{i=1}^{N}\left(\log_{10} y_i - \overline{\log_{10} y}\right)^2} = 1 - \frac{SSE}{SST}$$

where y_i is the measured effect concentration of sample i, $\hat{y}_i$ is the predicted effect concentration, $\overline{\log_{10} y}$ is the average of the log of the measured values, SSE is the sum of square errors, and SST is the total sum of squares.

To calibrate each parameter, the data were run through the BLM in speciation mode to obtain the critical amount of Ni accumulation on the biotic ligand. Then, for each calcium, magnesium, or hardness series, the critical accumulation (CA) of the point with the minimum concentration of Ca, Mg, or hardness was selected for the toxicity run, as appropriate. The minimum value was used in calibration so that the Ca or Mg response alone were being calibrated (see Figure 3A) rather than having the slope and overall magnitude of the response change with each new log K value. The data were then run through the BLM in toxicity mode with the expert option enabled in order to get the predicted effect concentrations. These predicted values were compared to the reported values with the logR2 to select the best-fitting log K value. This procedure is repeated for Ca and Mg iteratively until the optimum log K values remain constant.

Effects of hardness on Ni toxicity

As has been widely observed previously, increasing hardness reduces Ni toxicity and bioavailability, resulting in higher effect concentrations. Figure 4 summarizes the results from acute exposures with invertebrate organisms (Figure 4A) and fish (Figure 4B). Very similar patterns of increasing Ni effect concentrations with increasing hardness are evident for invertebrates and fish. The slope of the hardness effect was typically steeper in exposures that co-varied pH and alkalinity (e.g., Chapman et al 1980), than studies that varied hardness alone (e.g. Keithly et al 2004), and this difference in expected hardness

slope is consistent with BLM predictions for these studies, since experiments that vary alkalinity and pH along with hardness include the protective effects of increasing Ni-bicarbonate complexes in addition to the competition from calcium and magnesium ions that occur when hardness is varied alone.

The acute US EPA water quality criterion, also called the criterion maximum concentration or CMC, is based on a log-log equation dependent on hardness (US EPA, 1986) using an equation with the form:

$$CMC = e^{0.846[\ln(hardness)]+2.255}$$

The acute criterion is protective for most acute studies based on invertebrates, with the exception being *C. dubia* (Figure 4A) and is protective for all acute studies based on fish (Figure 4B).

The predicted BLM responses to hardness cations includes effects from both Ca and Mg and are determined by the Log K values for Ca and Mg binding to biotic ligand sites. The original Ni BLM calibration included only effects from Ca based on the limited information available at the time (Wu et al, 1986). For the present review, protective effects from both Ca and Mg are considered. To determine the appropriate log K values for Ca and Mg binding to the Ni BL site, the overall model behavior with varying log K values was compared with experimental results showing the protective effects from either cation alone or in combination.

The calibration for the calcium log K used data from Deleebeeck et al (2007b), Deleebeeck et al (2008), Kozlova et al (2009), and Meyer et al (1999) in which calcium alone was varied. Each of these studies individually calibrated to get optimum BL-Ca log K values at 3.80, 3.25, 4.50, and 4.05, respectively (see results in Figure 3B). Looking at the fit of all four studies collectively gives an optimum log K value at 4.25, which is the final log K value for the BL-Ca reaction that was decided upon. These calibrations were all done with a BL-Mg log K of 3.60.

The calibration for the magnesium log K only used data from Deleebeeck et al (2007b), Deleebeeck et al (2008), and Kozlova et al (2009), since Meyer et al (1999) did not perform a magnesium series. These three studies were calibrated to get optimum BL-Ca log K values of 3.75, 3.40, and 3.55, respectively (see results in Figure 3C). If the studies were once again calibrated collectively, the resulting optimum log K was 3.60, which was again used as the final log K for the BL-Mg reaction. These calibrations were all done with a BL-Ca log K of 4.25.

For the Ca and Mg Log Ks of the competitive interaction with Ni at BL sites were selected based on the logR2 results and correspond to 4.25 and 3.60 respectively (Table 2). The Ni BLM with these Log K values was able to predict Ni bioavailability with changing hardness conditions in acute exposures for both invertebrates (Figure 4A) and fish (Figure 4B). The same values for Ca and Mg Log Ks work well in chronic exposures based on lethal (Figure 5A) or sublethal (Figure 5B, 4C) endpoints that include variation in hardness to over 800 mg/L as $CaCO_3$.

As was noted with the acute Ni criterion, the US EPA chronic water quality criterion, also called the criterion continuous concentration or CCC, is based on a log-log equation dependent on hardness (US EPA, 1986) using an equation with the form:

$$CCC = e^{0.846[\ln(hardness)]+0.0584}$$

Both the acute and chronic US EPA criteria for Ni have the same slope for considering how the criteria should vary with hardness, and the similarity of the hardness effects in acute and chronic exposures as shown in Figure 4 (A and B) and Figure 5 (A, B, and C).

Effects of Ca on Ni toxicity

The effects of Ca alone, without co-variation in Mg or other cation concentrations, was investigated in five studies with invertebrates and fish (Figure 6). The effects of Ca on Ni toxicity in acute exposures to invertebrates were reported by Deleebeeck et al (2008) to *D. magna* and Kozlova et al (2009) to *D. pulex* (Figure 6A). In general, Ni toxicity in acute exposures to both invertebrates decreased with increasing Ca concentrations but for *D. magna* the protective effects of Ca are observed at Ca concentrations up to about 100 mg/L. At concentrations above 100 mg/L (with a corresponding hardness of 280 mg/L as $CaCO_3$) there does not appear to be any additional benefit of added Ca in the *D. magna* toxicity data reported by Deleebeeck et al (2008). For *D. pulex*, Kozlova et al (2009) reported protective effects up to about 60 mg/L.

A protective effect of increasing Ca on Ni toxicity was also reported in two studies with fish. Meyer et al (1999) reported a protective effect of Ca from 5 to about 100 mg/L in acute exposures with *P. promelas*. Deleebeeck et al (2007b) reported a protective effect of Ca from 4 to 40 mg/L in chronic exposures to *O. mykiss* and then no additional protective effect from 40 to 110 mg/L Ca.

The Ni BLM using a Log K of 4.25 for the competitive binding of Ca on Ni biotic ligand sites fit the protective response of added Ca well for most of the studies. However, for *D. magna* the protective benefit of added Ca reported by Deleebeeck et al (2008) is lower than expected based on the overall calibration to all studies (Figure 6A). In contrast, acute exposures with *D. magna* reported by Chapman et al (1980), which varied both Ca and Mg simultaneously, match the response of increasing hardness predicted by the BLM very well (Figure 4A). The two studies by Deleebeeck are the only two studies that show reduced or no protective benefit of added Ca at higher concentrations. This may be because few studies looked at Ca effects at concentrations above 100 mg/L. The only other study in this review that reported protective benefits of high concentrations of Ca was the Parametrix (unpublished) study with *C. dubia* that showed protective benefits consistent with the response predicted by the BLM to Ca concentrations of 237 mg/L. Another possible reason for the difference in observed effect of Ca on Ni toxicity seen in both of the Deleebeeck studies is that in these studies a Ca salt ($CaCl_2$) was added to soft water with a Mg concentration of around 5 mg/L to produce a wide range of Ca concentrations. As a result of this single salt addition, the Ca to Mg ratio becomes increasingly large as Ca concentrations increase. At 100 mg/L Ca and above the Ca:Mg ratio (in mg/L units) ranged from 19 to 34 in Deleebeeck et al (2008) and at 110 mg/L Ca the the Ca:Mg ratio was 37 in Deleebeeck et al (2007b). These high Ca:Mg ratios are much higher than other studies in this review, and are much than typical ratios seen in natural waters. For example, surface waters in North America typically range from 1.2 to 4.2 (10^{th} to 90^{th} percentile) with a median value of 2.

Deleebeeck et al (2008) noted that high concentrations of Ca introduced an additional stress in their study and for that reason the three highest Ca concentrations were excluded from subsequent analyses. An alternative explanation is that the reduced benefit of added Ca at high concentrations observed in the two studies by Deleebeeck et al (2007a, 2008) may be due to the unusual Ca:Mg ratios that resulted from the experimental design rather than the high concentrations of Ca. Other studies in this review avoided high Ca:Mg ratios either because both cations were allowed to vary to maintain a more constant ratio (Pickering 1974; Chapman et al 1980; Pyle et al, 2002; Keithly et al, 2004; Deleebeeck et al, 2007a; Parametrix – unpublished) or because variation in Ca was investigated over a smaller range of concentrations (Meyer et al 1999; Kozlova et al 2009). The possibility that the protective effects of Ca may be limited by unusual Ca:Mg ratios would explain why a consistent response to increasing Ca alone (Figure 6) or increasing Ca and Mg (Figures 2, and 4) is seen all studies included in this review where high Ca:Mg ratios were avoided.

Given that the protective effect of Ca seen in Deleebeeck et al (2008) is lower than that observed in other studies it is not surprising that the calibrated Log K of Ca binding to Ni biotic ligand sites reported by Deleebeeck et al (2008) is lower than the value suggested by this review (Table 2). The value of 4.25 used in this review is very close to the previous value used in development of the Ni BLM (Wu et al., 2003) which was based on measured Ni accumulation and measured competition between Ca and Ni in gill tissue reported by Meyer et al (1999).

Effects of Mg on Ni toxicity

The effects of Mg alone, without co-variation in Ca or other cation concentrations, was investigated in three studies with invertebrates and fish (Figure 7). The availability of studies that quantify Mg effects separately from Ca for this review is particularly useful since previous versions of the Ni BLM software included only Ca effects. In a similar experimental design as was used to investigate Ca effects, Deleebeeck et al (2008) and Kozlova et al (2009) quantified Mg effects on Ni toxicity to *D. magna* and *D. pulex* (Figure 7A) and Deleebeeck et al (2007b) quantified Mg effects on Ni toxicity to *O. mykiss* (Figure 7B).

As was noted in the Ca experiments, the organism response in the Mg experiments across these three studies showed a consistent reduction in Ni toxicity with increasing Mg at low to moderate Mg concentrations (Figure 7). The protective effect of Mg to invertebrate species reported by Deleebeeck et al (2008) and Kozlova et al (2009) were nearly identical at Mg concentrations less than 1 up to about 40 mg/L (Figure 7A), but at concentrations from 66 to 110 mg/L no additional protective effect to *D. magna* was observed. For *O. mykiss* a protective effect of Mg was observed from 3 to ~50 mg/L, but no additional protective effect was seen at concentrations above 50 mg/L (Figure 7B). For comparison the only other study found in this review that quantified Ni toxicity over this range of Mg concentrations was Parametrix (unpublished), which quantified toxicity to *C. dubia* at Mg concentrations that ranged from 3.7 to 78 mg/L. Over this range the protective effect of Mg and co-varying Ca continued to increase (Figure 5C). As was noted in the Ca experiments, the experimental manipulation of Mg without co-variation of Ca concentrations can lead to unusual Ca:Mg ratios. At the highest Mg concentrations used by both Deleebeeck et al (2008) and Deleebeeck et al (2007b) the Ca:Mg ratios were less than 0.1. Other studies in this review avoided similarly low Ca:Mg ratios by either adjusting both Ca and Mg together or by adjusting Mg over a narrower range of concentrations.

Previous versions of the Ni BLM software did not include Mg effects, since Mg was not explicitly considered in the accumulation data reported by Meyer et al (1999) used in model calibration documented by Wu et al. (2003). The value calibrated in this review, which considers the toxicity trends in the Mg-only and Mg+Ca experimental data (i.e., Figures 2 and 6) results in a Log K of 3.5 for Mg binding to the Ni BLM.

Effects of pH on Ni toxicity

The effects of pH on Ni toxicity in acute exposures were studied in three invertebrate studies and two fish studies (Figure 8). Both Deleebeeck et al (2008) and Kozlova et al (2009) investigated pH effects with and without the presence of a buffering agent. Buffering agents such as 3-morpholinepropanesulfonic acid (MOPS) are commonly used to control pH in metal toxicity studies and have been recommended for this purpose based on the fact that they do not affect metal speciation (Kandegedara and Rorabacher,

1999). Deleebeeck et al (2008) reported very little change in Ni toxicity to *D. magna* over the pH range 5.7 to 8.1, and this pattern was consistent whether or not MOPS was used in the exposures (Figure 8A). Kozlova et al (2009) also saw very little change in Ni toxicity to *D. pulex* over the pH range 5.6 to 8.3 in acute exposures without MOPS. A very different pattern, however, was reported by Kozlova et al (2009) in pH exposures with MOPS such that Ni EC50s increased with increasing pH (Figure 8A). It is unclear if the different patterns reported by these two studies are due to differences in how these two species respond to pH effects on Ni bioavailability. The fact that *D. magna* and *D. pulex* are closely related (in the same genus), and the similarity of their response to other factors compared in this review suggests that differences with respect to how MOPS may affect Ni bioavailability would be unlikely. The similarity of the reported response with and without MOPS in the tests reported by Deleebeeck et al (2008) may simply be due to the fact that MOPS was included in a relatively narrow range of pH conditions (5.7 to 6.6), whereas Kozlova et al (2009) investigated a wider range of pH conditions in tests with MOPS (i.e., 5.6 to 8.3), and the greatest differences in the pH response with and without MOPS were observed at pH values above 6.5.

Although MOPS and other pH buffers are commonly used in metal toxicity studies to help control pH, there is some controversy as to whether these compounds affect metal bioavailability. Esbaugh et al (2014) showed that the effects of changing pH on Pb toxicity to fathead minnow were different when either MOPS or enriched CO_2(g) environments were used to control pH conditions. These differences were attributed to physiological stress caused by changes in the pH gradients in apical gill membranes in fish in the presence of MOPS (Esbaugh et al., 2014). Since these buffers do not represent conditions in the natural environment, Esbaugh et al (2014) recommend that bioavailability data for metals determined in the presence of buffers should be avoided. Avoiding Ni bioavailability data in the presence of MOPS may be especially prudent since the comparison reported by Kozlova et al (2009) show inconsistent pH effects were observed in the presence of MOPS. To the extent that Ni toxicity data reported in exposures that include either MOPS or enriched CO_2(g) environments show different pH responses than natural waters or synthetic waters that more closely resemble natural conditions may be due to experimental artifacts that result from physiological stress and may not be relevant for the purpose of developing bioavailability models. Data from exposures that include either MOPS or enriched CO_2(g) environments are identified in Figure 8A and in the comments section of the supplemental data table to facilitate caution in the use of these data in subsequent model evaluations.

If only the data from Kozlova et al (2009) that do not include MOPS are considered, then there is essentially no pH effect on Ni toxicity observed for *D. pulex* over the pH range of 5.6 to 8.3, and this lack of a pH response is consistent with the predicted trend using the Ni BLM (Figure 8A) using the parameters in Table 2. The pH effect observed by Deleebeeck et al (2008) with *D. magna* showed at most a minor increase in EC50 over the pH range 5.7 to 8.1. Although the EC50s are a little higher than predicted by the Ni BLM they are still within a factor of 2 and consistent with the trend predicted by the model, which is that little if any pH effect to invertebrates is expected from pH 5.6 to 8.3.

In contrast, Schubauer-Berigan et al (1993) reported an approximately 10-fold decrease in Ni EC50s in acute exposures to *C. dubia* from pH 7.3 to 8.7 (Figure 8B). This result is unusual given the lack of a strong pH effect with other invertebrates (Figure 8A), which if anything suggested a slight increase in Ni EC50s over a much larger pH range. The experimental conditions of Schubauer-Berigan et al were also unusual in that the test chambers were sealed to prevent gas exchange after pH adjustments were made. If gas exchange was effectively prevented, these conditions would result in a CO_2(g) enriched environment in the samples with lower pH relative to the exposure at pH 8.7. It is unclear whether the unusual pH response is due to these experimental conditions, or to species-specific differences in how pH affects Ni bioavailability to *C. dubia*. The Ni BLM can be made to predict lower EC50s at high pH by

adjusting the NiOH Log K from -5.5 to -4.0 (dashed line on Figure 8b), but the uniqueness of this response, the fact that is has only been observed in one sample, and the possibility that it may be due to experimental conditions, suggests that it should be replicated in other tests with *C. dubia* prior to adopting an alternate model calibration.

Schubauer-Berigan et al (1993) in acute exposures with *P. promelas* reported no pH effect over the range 7.3 to 8.7 (Figure 8C). Pyle et al (2002) also did not see a strong pH effect in acute exposures with *P. promelas*, although approximately a three-fold increase in Ni LC50s were reported over the pH range 5.5 to 8.5 (Figure 8C). The lack of a strong pH effect in acute studies with fish is consistent with the pH effects seen in all but one of the acute studies with invertebrates. The consistency of the pH response in exposures not affected by MOPS or enriched CO_2(g) environments suggests that a Ni BLM with a single set of parameters will fit both invertebrates and fish. The similarity in the observed pH response for fish and invertebrates further reinforces the notion that the unique response reported by Schubauer-Berigan et al (1993) for *C. dubia* should be replicated prior to deciding whether it should be used in the development of a bioavailability model for Ni.

Another study that showed atypical effects of pH on Ni toxicity was a chronic test with *O. mykiss* reported by Deleebeeck et al (2007b), which shows increasing Ni LC50s at pH values lower than 7.5, and with little pH effect from 7.5 to 8.5 (Figure 9). This was the only study that indicated possible proton competition at pH values below 7, and as a result the higher log K for the BL-H binding used in the original calibration (Wu et al., 2003) was a better fit than the revised value (Table 2). The BLM prediction using the lower log K for the BL-H binding value consistent with the acute tests with invertebrates and fish results in an overestimation of toxicity at low pH, compared with the with *O. mykiss* LC50s reported by Deleebeeck et al (2007b) as shown with the solid line in Figure 9. The dashed line in Figure 9 uses the higher log K for the BL-H binding (Wu et al., 2003) and more closely matches the trends in the chronic toxicity data for *O. mykiss*. However, this is the only test and only organism which suggests this level of competitive effects of protons is appropriate. It is also the only chronic test with a fish that looked at pH effects. Since the pH response seen in this study is unlike all other studies, replication of this result would be prudent, prior to recommending the higher Log K value. It should also be noted that the lowest pH exposure included MOPS buffer, and as already discussed, MOPS may alter the bioavailability of Ni and other metals.

Effects of Dissolved Organic Carbon on Ni toxicity

Three studies investigated the effects of natural organic matter (NOM) on Ni toxicity. Kozlova et al (2009) used two different organic matter sources, Suwannee River NOM (SRNOM) and Nordic Reservoir NOM NRNOM), in acute tests with *D. pulex*. Both sources of NOM, quantified by measurement of dissolved organic carbon (DOC), reduced the toxicity of Ni (Figure 10A). The effect of either type of NOM was consistent from 0 to 10 mg/L DOC showing a reduction in toxicity (higher LC50s) with higher DOC. At concentrations above 10 mg/L the effect of increasing amounts of NRNOM reduced Ni toxicity further, while SRNOM did not have an additional protective effect at concentrations above 10 mg/L. From 1 to 40 mg/L DOC, the Ni LC50s to *D. pulex* increased about 7-fold when NRNOM was added, and only about 3-fold when SRNOM was used (Figure 10A).

When similar concentrations of NOM were added to toxicity tests with a less sensitive organism, a smaller overall effect on Ni toxicity was seen. For example, Doig and Liber (2006) reported the effects of five different NOM sources on *H. azteca* including whole peat (WP), peat hydrophilic DOC (PHD), peat fulvic acid (PFA), Suwanee River fulvic acid (SRFA) and Suwanee River humic acid (SRHA). Although the range of DOC concentrations added (i.e., 0-30 mg/L) was similar to that of Kozlova et al (2009) a less than 2-fold increase in Ni LC50s was observed (Figure 10A). Doig and Liber (2006) did not see

differences in the effect of NOM on Ni bioavailability and therefore concluded that the quantity of NOM was more important than the quality of the NOM.

The effects of NOM are simulated in the BLM using a set of discrete binding sites calibrated for proton and metal binding calibrated in the development of the WHAM model (Tipping 1994). The reactions developed for WHAM are simulated in the BLM as part of the overall conceptual model dealing with metal bioavailability such that NOM can bind Ni and other metals, thereby reducing the chemical activity of the metal and reducing the extent to which it can bind to biotic ligand sites (Figure 1). These reactions include metal complexation at sites with a range of binding strengths representative of different types of reactive functional groups found in NOM.

BLM simulations of the expected effects of NOM on Ni bioavailability match the overall trends observed by Kozlova et al (2009) for *D. pulex* with NRNOM addition from 1 to 35 mg/L (Figure 10A). As previously noted the effects of SRNOM addition were similar to NRNOM from 1 to 10 mg/L DOC and in this range observed effects agree well with BLM predictions. No additional protective effect was observed from SRNOM additions at DOC concentrations from 20 mg/L – 40 mg/L and in this DOC range; however the BLM predicts that additional protective effect should be expected in a manner consistent with the effects observed in the NRNOM addition (Figure 10A).

The BLM predictions for *H. azteca* match the effects of NOM additions reported by Doig and Liber (2006) over a DOC range of 0 to 35 mg/L (Figure 10A), including the observation that NOM effects to this organism are smaller than that observed for *D. pulex*. Since the BLM includes reactions with a range of NOM binding sites, complexation reactions at low metal concentrations are dominated by interactions with strong binding sites. As metal concentrations increase, the strongest binding sites become saturated with metal thereby shifting the binding of added metal to the next strongest set of binding sites. As a result, the overall strength of metal-NOM interactions is dependent on the relative concentrations of metal and NOM. As a result, the BLM predicts larger NOM effects in conditions that are associated with lower metal concentrations (e.g., more sensitive organisms or life stages) compared with conditions associated with higher metal concentrations (e.g., less sensitive organisms or life stages). This concentration-dependent behavior is illustrated by the relatively steeper slope of the BLM predicted response to NOM additions for *D. pulex* compared with *H. azteca* and is consistent with reported observations in Kozlova et al (2009) and Doig and Liber (2006) (Figure 10A).

The effects of NOM on Ni toxicity were one of several water quality parameters investigated by Hoang et al (2004) in acute exposures to *P. promelas*. Several additional toxicity tests using the same experimental design but not included in the Hoang et al (2004) study are included here (see Supplemental table). Hoang et al (2004) concluded that Ni toxicity was affected by fish age, DOC, pH, hardness, and alkalinity and those findings are consistent with the results of the Ni BLM. The Ni BLM was able to predict variation in toxicity to *P. promelas* over a wide range of pH, hardness, alkalinity, and DOC concentrations (Figure 10b).

Validation of the Ni BLM in synthetic and natural waters

Throughout this analysis, the focus has been on comparing the Ni BLM to experiments where a single water quality factor has been adjusted. Experiments where one or more water quality factors change are also useful for model evaluation. Summaries of model performance against all synthetic waters used for acute and chronic Ni toxicity tests are shown in Figure 11. The acute summary (Figure 11A) contains all of the acute studies listed in Table 1, except for pH exposures where MOPS were added and the high Ca (Ca > 100 mg/L) and high Mg (Mg > 50 mg/L) tests of Deleebeeck et al (2007b).

Throughout this analysis, the Ni BLM was applied with a consistent set of parameters including Log K values (Table 2) and species mean effect accumulations (SMEA; Table 3). The SMEAs are similar to the lethal accumulation (or LA50) term used in previous BLM modeling but this change in name is more consistent with the wide variety of lethal and sub-lethal endpoints in acute and chronic exposures used in recent BLM evaluations.

The values of SMEAs (Table 3) used in this analysis provide a measure of the sensitivity of various organisms, endpoints, and lifestages, such that lower SMEAs indicate greater sensitivity. In general, invertebrates represent the most sensitive species in both acute and chronic exposures. For organisms where information for multiple lifestages is available, such as for *P. promelas*, younger and smaller fish are more sensitive than older and larger fish and have correspondingly smaller SMEAs as a result.

The overall comparison of Ni BLM predictions for organisms in synthetic waters shows excellent agreement with nearly all predictions within a factor of two of measured values (Figure 11). Agreement within a factor of 2 has traditionally been used to indicate good performance (Di Toro et al., 2001). Recently this level of agreement was shown to correspond to the variability observed in replicate toxicity tests represented by approximately 1.5 standard deviations around the median (Santore and Ryan, 2015). This level of agreement between predicted and measure values, therefore, is comparable to the level of agreement expected between replicate measurements. One data point that falls well away from this acceptable level of agreement is the acute *C. dubia* LC50 reported by Schubauer-Berrigan at pH 8.7. Other studies that report *C. dubia* data show much better correspondence with Ni BLM predictions such as acute data from Keithly et al (2004) and chronic data from Keithly et al (2004) and Parametrix (unpublished), even when data from these other studies include observations at similar pHs. The lowest *C. dubia* LC50 reported by Schubauer-Berrigan, in this context, does seem to be anomalously low for an acute test, and is more reflective of lowest of the range of values observed in chronic *C. dubia* endpoints (Figure 11B).

Since many of the same datasets for Ni toxicity in synthetic waters used in the overall comparisons shown in Figure 11A,B were also used for calibration, this level of overall goodness of fit should be expected. Application of the Ni BLM to an independent set of toxicity tests in natural waters shows a similar level of excellent agreement in acute exposures (Figure 12A). Lind et al. (1978) measured Ni toxicity to *D. pulicaria* in samples from lakes and rivers that covered wide ranges in pH (5.8 to 8.1), DOC (2.6 to 39 mg/L), and hardness (25 to 120 mg/L as $CaCO_3$). The Ni BLM was able to accurately predict Ni toxicity over this wide range of conditions (Figure 12A), although for the three samples from Lake Superior the BLM predicted LC50s that were consistently low (see Supplemental Table). The Lake Superior samples were the highest pH conditions in the range of samples which indicate that for *D. pulicaria*, Ni toxicity may be reduced at high pH. Lind et al. (1978) also reported LC50s for *P. promelas* and for these tests the Ni BLM predicts LC50s that match measured values well including at high pH (Figure 12A). Deleebeeck et al (2008) reported Ni EC50s from eight sites in Europe that covered wide ranges in pH (5.9 to 8.1), DOC (1.8 to 26 mg/L), and hardness (13 to 266 mg/L as $CaCO_3$) to *D. magna* and the Ni BLM performed well over these wide ranges of conditions (Figure 12A).

In chronic exposures Deleebeeck et al (2007b) reported Ni LC50s for *O. mykiss* in samples from five sites. The Ni BLM performed well for all five natural waters. Although the natural waters had variation in pH from 5.6 to 8.2 there was no suggestion that the natural waters results indicated that there was a competitive interaction between Ni and protons, similar to what was seen in the synthetic waters tests from the same study (Figure 9). Parametrix (unpublished) reported IC25s for *C. dubia* in six natural waters, and while the Ni BLM predicted IC25 values close to what was measured, the model predicted higher IC25s for samples from the Grand River, which had the highest DOC (near 7.5 mg/L), even though

measured IC25s at this site were among the lowest of the natural waters in this study (Figure 12B and Supplemental Table).

Schlekat et al (2010) reported chronic Ni toxicity to four different species in four natural waters. One of the natural waters (S. Platte) was tested at ambient pH and at an acidified pH which was adjusted by equilibration with an elevated CO_2(g) environment. As previously noted, elevated CO_2(g) environments may result in additional physiological stress to test organisms (Esbaugh et al 2014), and the pH adjusted samples are identified by dashed circles around individual data points on Figure 12B to allow for comparison. However, of the four species, only the *B.calyciflorus* prediction showed a large deviation from the reported value in this acidified sample. All four of these species had not previously been tested for Ni toxicity and as a result there were no previous calibrations of the Ni BLM to these organisms. Of the four species tested, the Ni BLM predicted EC20s or EC50s to *C. tentans* and *L. minor* in good agreement with measured values (Figure 12B). For *B. calyciflorus*, however, one of the five samples resulted in a large discrepancy between measured and modeled values; subsequent tests by Schlekat et al (2010) concluded that the toxicity in this sample was not due to Ni and it was removed from further consideration in their analysis. The Ni toxicity data to *L. stagnalis* reported by Schlekat et al (2010) was the only organism in this study that showed a different pattern than was expected according to the Ni BLM. Predicted toxicity in these samples deviated from observations such that predictions in high pH samples were higher than what was observed. For *L. stagnalis*, therefore, Ni toxicity may increase at high pH, making this the only dataset found in this review other than the *C. dubia* data reported by Schubaer-Berrigan that suggests increased toxicity at high pH.

Summary and conclusions

The Ni BLM using a single set of parameters was able to successfully predict the modifying effects of water chemistry on Ni bioavailability and toxicity to a wide variety of fish and invertebrates in acute and chronic exposures. Nickel toxicity was shown to be modified by a number of water quality factors including Ca, Mg, and the presence of natural organic matter and these effects were consistent for both fish and invertebrates. The consistency of these effects allowed a single set of BLM parameters (Table 2) to fit observations from a wide array of organisms in across a wide range of water chemistries in both acute and chronic tests.

The effects of pH on Ni toxicity did not appear to be consistent across all organisms. Most invertebrates and fish for which pH trends have been reported, showed very little variation in Ni bioavailability across a wide range of pH values, or a slight reduction in toxicity at pH values above 8 (Figure 8). The exceptions appear to be *O. mykiss*, *C. dubia*, and *L. stagnalis*. Tests with *O. mykiss* showed reduced toxicity at low pH in synthetic waters (Figure 9). The trend for *O. mykiss* in Figure 9 indicated that higher LC50 values were observed at low pH, and the trend leveled off from neutral pH to pH 8.5. This pattern is distinctly different from that seen in *C. dubia* which were continued to decrease at pH 8.5 and higher (Figure 8B). Hence, the *O. mykiss* trend is described as elevated at low pH, rather than reduced at high pH. However this same trend was not evident in natural water tests. For natural waters, chronic exposures with *O. mykiss* were well described by the Ni BLM over a range of pH from 5.6 to 8.2 (Figure 12B). The reason for the difference in behavior in synthetic waters and natural waters reported by Deleebeeck et al (2007b) is not clear, however the lowest pH exposure in the synthetic water series did include MOPS buffer, and other tests included in this review indicate that MOPS can affect the pH effect observed in Ni bioavailability studies (Figure 8A; Kozlova et al., 2009). Although buffers such as MOPS were designed to have no effect on metal speciation (Kandegedara and Rorabacher, 1999), they have been shown to

affect metal bioavailability via alteration of the chemical microenvironment near biological membranes (Esbaugh, et al 2014). Differences observed on the effects of pH on the bioavailability of Ni in exposures with and without MOPS provide further evidence that these buffers may have unintended impacts when used in metal bioavailability studies.

Despite the differences noted in the pH trends for some organisms, the overall patterns of behavior of Ni bioavailability suggest that there are far more similarities than differences when comparing the factors that control Ni bioavailability in natural waters across wide ranges of water chemistry. These common bioavailability factors affect Ni toxicity in acute and chronic exposures to fish and invertebrate species, and suggest that a unified framework for addressing bioavailability effects such as the Ni BLM could be used to predict Ni toxicity in risk assessment and regulatory settings.

References

Chapman, G.A, S. Ota, and F. Recht. 1980 Manuscript. Effects of water hardness on the toxicity of metals to *Daphnia magna*. U.S. EPA, Corvalis, Oregon.

Deleebeeck, N.M.E., B.T.A. Muyssen, F. De Laender, C.R. Janssen, and K.A.C. De Schamphelaere. 2007. Comparison of nickel toxicity to cladocerans in soft versus hard surface waters. Aquatic Toxicology, vol 84, pp. 223-235.

Deleebeeck, N.M.E., K.A.C. De Schamphelaere, and C.R. Janssen. 2007. A bioavailability model predicting the toxicity of nickel to rainbow trout (*Oncorhynchus mykiss*) and fathead minnow (*Pimephales promelas*) in synthetic and natural waters. Ecotoxicology and Environmental Safety, vol 67, pp.1-13.

Deleebeeck, N.M.E., K.A.C. De Schamphelaere, D.G. Heijerick, B.T.A. Bossuyt, and C.R. Janssen. 2008. The acute toxicity of nickel to *Daphnia magna*: Predictive capacity of bioavailability models in artificial and natural waters. Ecotoxicology and Environmental Safety, vol 70, pp. 67-78.

Dominic M. Di Toro, Herbert E. Allen, Harold L. Bergman, Joseph S. Meyer, Paul R. Paquin, Robert C. Santore. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environmental Toxicology and Chemistry 11/2001; 20(10):2383-96.

Doig, L.E., and K. Liber. 2006. Influence of dissolved organic matter on nickel bioavailability and toxicity to *Hyalella azteca* in water-only exposures. Aquatic Toxicology, vol. 76, pp. 203-216.

Esbaugh, A.J., E M Mager, K V Brix, R Santore, M Grosell. 2014. Implications of pH manipulation methods for metal toxicity: Not all acidic environments are created equal. Aquatic toxicology (Amsterdam, Netherlands) 12/2012; 130-131C:27-30

Hoang, T.C., J.R. Tomasso, and S.J. Klaine. 2004. Influence of water quality and age on nickel toxicity to fathead minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry, vol. 23, Iss. 1, pp. 86-92.

Kandegedara, A, and D.B. Rorabacher. 1999. Noncomplexing Tertiary Amines as "Better" Buffers Covering the Range of pH 3-11. Temperature Dependence of Their Acid Dissociation Constants. Analytical Chemistry 71(15):3140-4

Keithly, J., J.A. Brooker, D.K. DeForest, B.K. Wu, and K.V. Brix. 2004. Environmental Toxicology and Chemistry, vol. 23, iss. 3, pp. 691-696.

Kozlova, T., C.M. Wood, and J.C. McGeer. 2009. The effect of water chemistry on the acute toxicity of nickel to the cladoceran *Daphnia pulex* and the development of the biotic ligand model. Aquatic Toxicology, vol 91, pp. 221-228.

Leonard, E.M., C.M. Wood. 2013. Acute toxicity, critical body residues, Michaelis–Menten analysis of bioaccumulation, and ionoregulatory disturbance in response to waterborne nickel in four invertebrates: *Chironomus riparius*, *Lymnaea stagnalis*, *Lumbriculus variegatus* and *Daphnia pulex*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. Volume 158, Issue 1, June 2013, Pages 10–21

Lind, D., K. Alto, and S. Chatterton. 1978. Regional Copper-Nickel Study: Aquatic Toxicology Study. Minnesota Environmental Quality Board.

Meyer, J.S., R.C. Santore, J.P. Bobbitt, L.D. Debrey, C.J. Boese, P.R. Paquin, H.E. Allen, H.L. Bergman, and D.C. Ditoro. 1999. Binding of Nickel and Copper to Fish Gills Predicts Toxicity When Water Hardness Varies, But Free-Ion Activity Does Not. Environmental Science and Technology, vol 33, pp. 913-916.

Parametrix (unpublished). Personal communication with Dr. William Stubblefield, Oregon State University.

Pickering, QH. 1974. Chronic toxicity of nickel to fathead minnow. Water Pollution Control Federation, vol 46, pp. 342–347.

Pyle, G.G., S.M. Swanson, and D.M. Lehmkuhl. 2002. The influence of water hardness, pH, and suspended solids on nickel toxicity to larval fathead minnows (*Pimephales promelas*). Water, Air, and Soil Pollution, vol 133, pp. 215-226.

Santore, R.C., D M Di Toro, P R Paquin, H E Allen, J S Meyer. 2001. Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. Environmental Toxicology and Chemistry 11/2001; 20(10):2397-402.

Santore, R.C., R. Mathew, P.R. Paquin, D. DiToro. 2002. Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. Comparative Biochemistry and Physiology Part C Toxicology & Pharmacology 10/2002; 133(1-2):271-85.

Santore, R.C., A.C. Ryan. 2015. Development and application of a multi-metal multi-biotic ligand model for assessing toxicity of metal mixtures. Environmental Toxicology and Chemistry, Environ Toxicol Chem 2015;34:777–787

Schlekat, C.E., E. Van Genderen, K.A.C. De Schamphelaere, P.M.C. Antunes, E.C. Rogevich, and W.A. Stubblefield. 2010. Cross-species extrapolation of chronic nickel Biotic Ligand Models. Science of the Total Environment, vol 408, pp. 6148-6157.

Schubauer-Berigan, M.K., J.R. Dierkes, P.D. Monson, and G.T. Ankley. 1993. pH-dependent toxicity of Cd, Cu, Ni, Pb, and Zn to *Ceriodaphnia dubia, Pimephales promelas, Hyalella Azteca, and Lumbriculus variegatus*. Environmental Toxicology and Chemistry, vol 12, pp. 1261-1266.

Tipping, E. 1994. WHAM—a chemical equilibrium model and computer code for waters, sediments, and soils incorporating a discrete site/electrostatic model of ion-binding by humic substances. Computers & Geosciences archive Volume 20 Issue 6, July 1994. Pages 973 - 1023

U.S. EPA, 1986. Ambient Water Quality Criteria for Nickel, EPA 440/5-86-004. U.S. Environmental Protection Agency,Washington, DC, USA.

Wu, K.B., Paquin, P.R., Navab, V., Mathew, R., Santore, R.C., Di Toro, D.M., 2003. Development of a biotic ligand model for nickel: Phase I. Report Number 01-ECO-10-T. Water Environment Research Foundation, Alexandria, VA, USA.

Figure 1. Conceptual model for the Ni BLM showing interaction between chemical speciation and accumulation of Ni on biological membranes. In the Chemical Speciation box, complexation reactions between natural organic matter (NOM) or inorganic ligands such as carbonate and hydroxide can determine the amount of free Ni ion, thereby affecting the amount of Ni accumulation on biotic ligand sites (BL-Ni). Accumulation of Ni can also be affected by competition with other cations such as calcium and magnesium.

Figure 2. SMEA-normalized Ni toxicity to aquatic invertebrates (A) and fish (B) in studies that varied hardness conditions. Individual symbols correspond to reported LC50 or EC50 values. Solid lines represent BLM predicted LC50 or EC50 values for corresponding conditions.

Figure 3. Graphical representation of the effects of changes in the BL-Ca log K (A), and variation in goodness of fit statistics with Log K values for either Ca (B) or Mg (C) binding to biotic ligand sites. Goodness of fit is determined by the correlation coefficient of the log-transformed data (i.e., log-R-squared). Statistics are summarized considering only individual studies with single ion tests (i.e, Ca in A or Mg in B) or all of the studies shown combined. Numbers closer to 1 are better for log-R-Squared.

Figure 4. Acute Ni toxicity to aquatic invertebrates (A) and fish (B) in studies that varied hardness conditions. Individual symbols correspond to reported LC50 or EC50 values. Solid lines represent BLM predicted LC50 or EC50 values for corresponding conditions. The dotted line represents the US EPA acute water quality criteria.

Figure 5. Chronic Ni toxicity to aquatic invertebrates based on survival (A) or reproduction (B, C) in studies that varied hardness conditions. Individual symbols correspond to reported LC20/50 (A), EC20/50 (B), or IC25 (C) values. In panels A and B solid lines represent BLM predicted values for corresponding conditions. In panel C measured IC25 values are shown as filled circles. BLM predictions shown as "+" were run for conditions in each test, in which more than just hardness varied. The dotted line in each panel represents the US EPA chronic water quality criteria.

Figure 6. Ni toxicity to aquatic organisms in studies that varied Ca alone in acute exposures to invertebrates (A) or fish (B), or chronic exposures to fish (C). Individual symbols correspond to reported toxicity values. Solid lines represent BLM predicted values for corresponding conditions.

Figure 7. Nickel toxicity to aquatic organisms in studies that varied Mg alone in acute exposures to invertebrates (A) or fish (B). Individual symbols correspond to reported toxicity values. Solid lines represent BLM predicted values for corresponding conditions.

Figure 8. Nickel toxicity to aquatic organisms in studies that varied pH in acute exposures to invertebrates (A, B) or fish (C). Individual symbols correspond to reported toxicity values. Solid lines represent BLM predicted values for corresponding conditions. The dashed line in B shows an alternative calibration to C. dubia that emphasizes increased bioavailability at high pH.

Figure 9. Nickel toxicity to aquatic organisms in studies that varied pH in chronic exposures to fish . Individual symbols correspond to reported toxicity values. Solid lines represent BLM predicted values for

corresponding conditions. The dashed line shows an alternative calibration to O. mykiss that emphasizes reduced bioavailability at low pH.

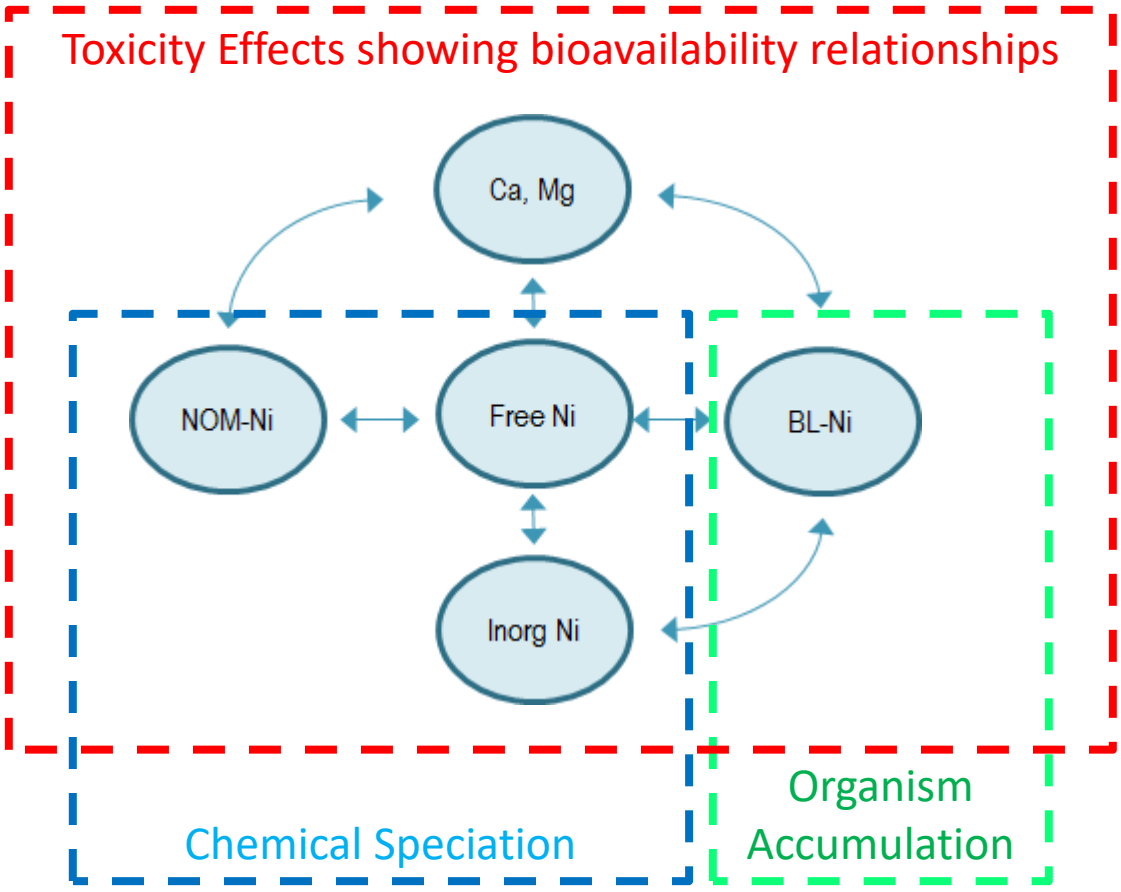
Figure 10. Nickel toxicity to aquatic organisms in studies that varied DOC in acute exposures to invertebrates (A) or fish (B). Individual symbols correspond to reported toxicity values. Solid lines represent BLM predicted values for corresponding conditions.

Figure 11. Overall performance with the best overall model for acute (A) or chronic (B) fish and invertebrate tests in synthetic waters. The solid black line shows perfect agreement between measured and predicted Ni toxicity and the dashed lines indicate plus or minus a factor of two away from perfect agreement.

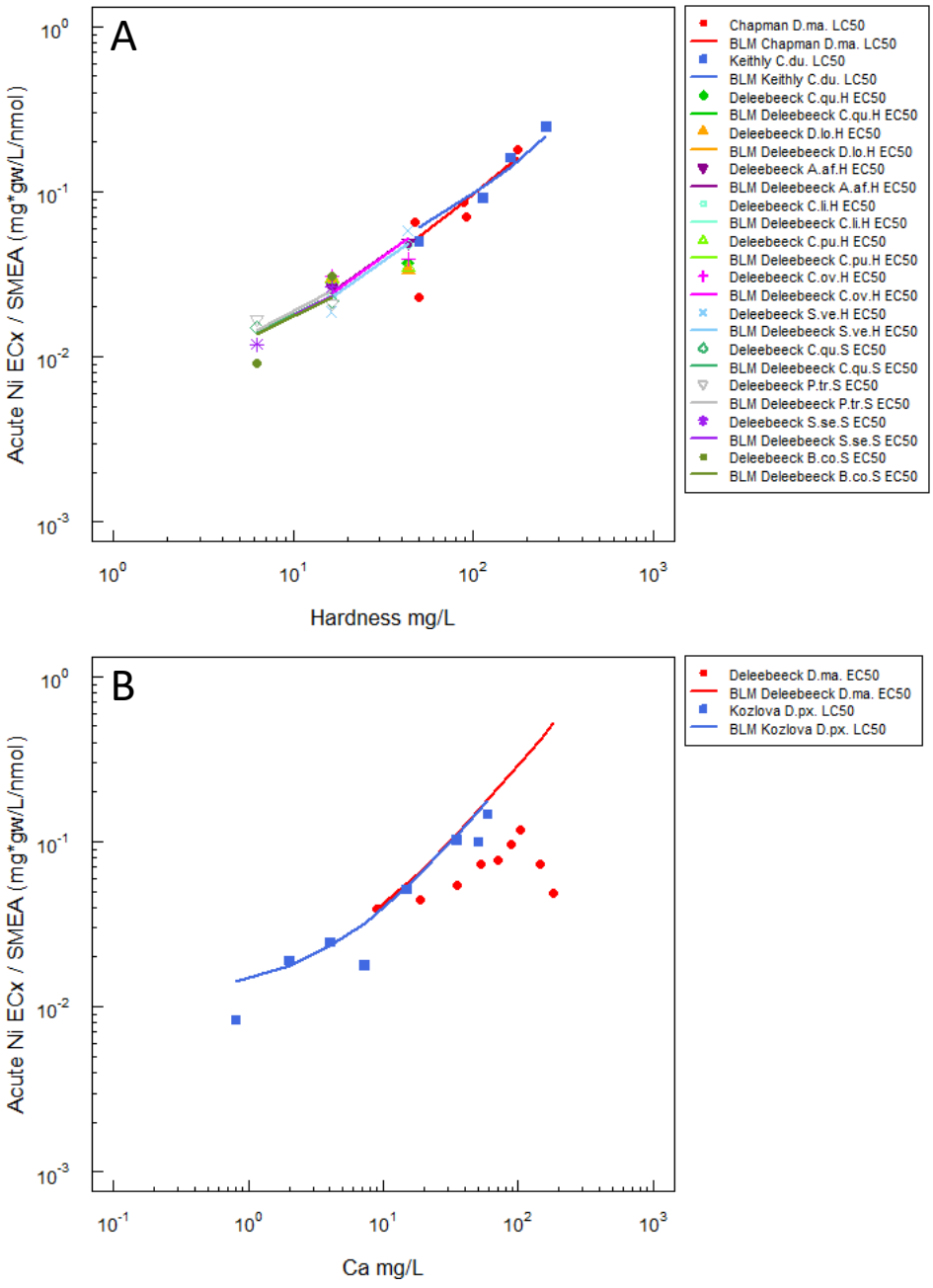
Figure 12. Overall performance with the best overall model for acute (A) or chronic (B) fish and invertebrate tests in natural waters. The solid black line shows perfect agreement between measured and predicted Ni toxicity and the dashed lines indicate plus or minus a factor of two away from perfect agreement.

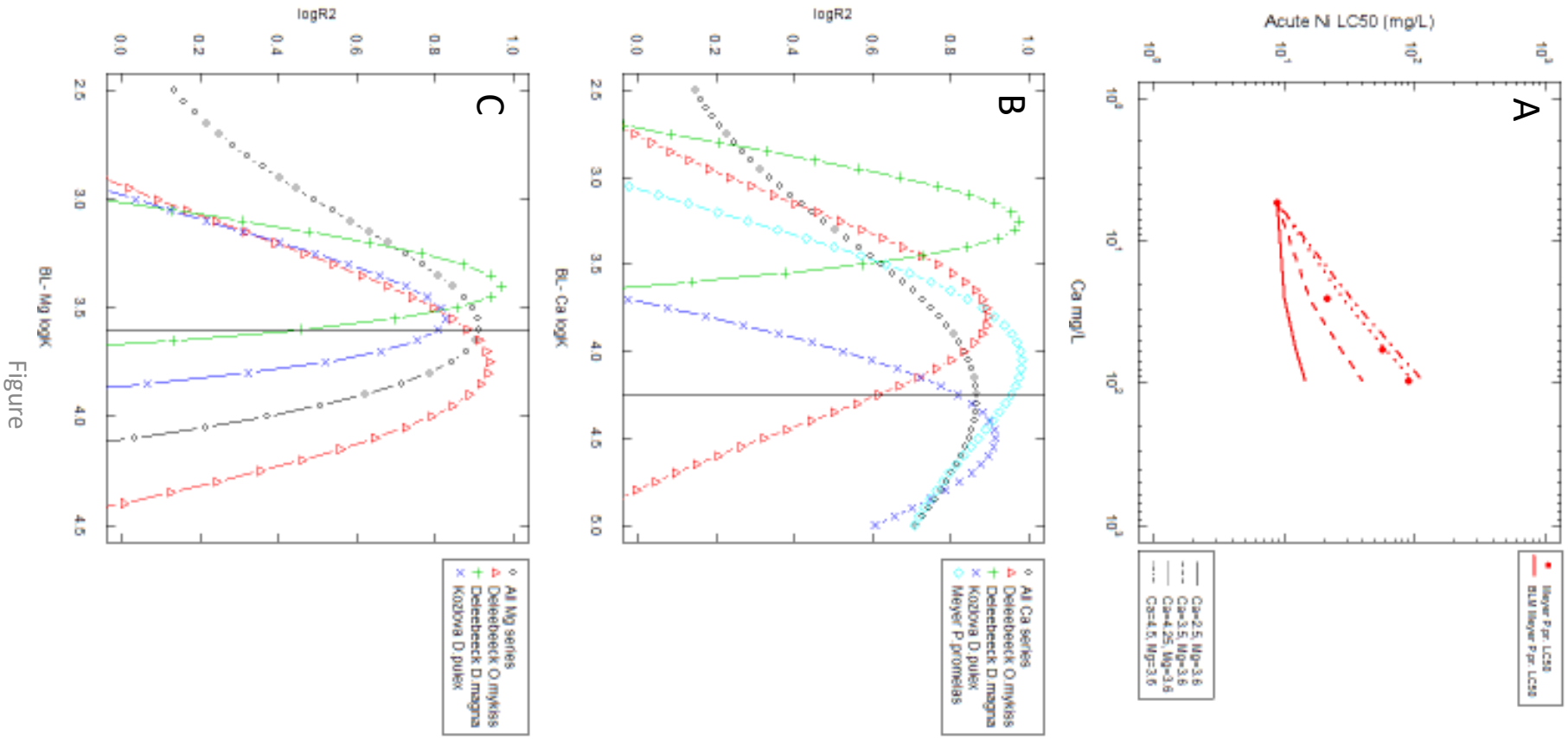
Species	Lifestage/Age	Exposure Duration	Reported Effect	Endpoint	# Obs	Study
Acute						
Alona affinis (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Bosmina coregoni (S)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Ceriodaphnia dubia	<1d	2d	LC50	survival	4	Keithly et al (2004)
Ceriodaphnia dubia	Not Reported	2d	LC50	survival	3	Schubauer-Berigan et al (1993)
Camptocercus lilljeborgi (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Chydorus ovalis (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Ceriodaphnia pulchella (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (S)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Daphnia longispina (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Daphnia magna	<1d	2d	EC50	immobilization	52	Deleebeeck et al (2008)
Daphnia magna	Not Reported	Not Rep.	LC50	survival	5	Chapman et al (manuscript)
Daphnia pulex	<1d	2d	LC50	immobilization	44	Kozlova et al (2009)
Daphnia pulicaria	Not Reported	2d	LC50	survival	16	Lind et al (1978)
Hyalella azteca	7-14d	2d	LC50	survival	20	Doig & Liber (2006)
Pimephales promelas	<1d	4d	LC50	survival	16	Hoang et al (2004 & unpublished)
Pimephales promelas	<1d	4d	LC50	survival	6	Pyle et al (2002)
Pimephales promelas	100d	4d	LC50	survival	4	Pickering (1974)
Pimephales promelas	28d ± 1	4d	LC50	survival	18	Hoang et al (2004 & unpublished)
Pimephales promelas	Not Reported	4d	LC50	survival	8	Lind et al (1978)
Pimephales promelas	Not Reported	4d	LC50	survival	3	Schubauer-Berigan et al (1993)
Pimephales promelas	subadult (1-6 g)	4d	LC50	survival	4	Meyer et al (1999)
Peracantha truncata (S)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Simocephalus serrulatus (S)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Simocephalus vetulus (H)	<2d	2d	EC50	immobilization	2	Deleebeeck et al (2007a)
Chronic						
Alona affinis (H)	<2d	16d	LC50	survival	2	Deleebeeck et al (2007a)
Brachionus calyciflorus	Not Reported	10d	EC10	population growth rate	5	Schlekat et al (2010)
Brachionus calyciflorus	Not Reported	10d	EC20	population growth rate	5	Schlekat et al (2010)
Ceriodaphnia dubia	<1d	7d	EC20	reproduction	3	Keithly et al (2004)
Ceriodaphnia dubia	<1d	7d	IC25	survival and reproduction	19	Parametrix (unpublished)
Ceriodaphnia dubia	<1d	7d	LC20	survival	3	Keithly et al (2004)
Ceriodaphnia pulchella (H)	<2d	17d	EC10	reproduction	2	Deleebeeck et al (2007a)
Ceriodaphnia pulchella (H)	<2d	17d	EC50	reproduction	2	Deleebeeck et al (2007a)

Ceriodaphnia pulchella (H)	<2d	17d	LC50	survival	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (H)	<2d	17d	EC10	reproduction	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (H)	<2d	17d	EC50	reproduction	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (H)	<2d	17d	LC50	survival	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (S)	<2d	17d	EC10	reproduction	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (S)	<2d	17d	EC50	reproduction	2	Deleebeeck et al (2007a)
Ceriodaphnia quadrangula (S)	<2d	17d	LC50	survival	2	Deleebeeck et al (2007a)
Chironomus tentans	Not Reported	10d	EC10	ash free dry weight	5	Schlekat et al (2010)
Chironomus tentans	Not Reported	10d	EC20	ash free dry weight	5	Schlekat et al (2010)
Daphnia longispina (H)	<2d	21d	EC10	reproduction	2	Deleebeeck et al (2007a)
Daphnia longispina (H)	<2d	21d	EC50	reproduction	2	Deleebeeck et al (2007a)
Daphnia longispina (H)	<2d	21d	LC50	survival	2	Deleebeeck et al (2007a)
Lemna minor	Not Reported	10d	EC10	growth rate	5	Schlekat et al (2010)
Lemna minor	Not Reported	10d	EC50	growth rate	5	Schlekat et al (2010)
Lymnea stagnalis	<1d	10d	EC10	wet weight	5	Schlekat et al (2010)
Lymnea stagnalis	<1d	10d	EC20	wet weight	5	Schlekat et al (2010)
Lymnea stagnalis	<1d	10d	EC50	wet weight	5	Schlekat et al (2010)
Onchorynchus mykiss	juvenile (28-35d)	17-26d	LC50	survival	20	Deleebeeck et al (2007b)
Peracantha truncata (S)	<2d	17d	EC10	reproduction	2	Deleebeeck et al (2007a)
Peracantha truncata (S)	<2d	17d	EC50	reproduction	2	Deleebeeck et al (2007a)
Peracantha truncata (S)	<2d	17d	LC50	survival	2	Deleebeeck et al (2007a)
Simocephalus serrulatus (S)	<2d	17d	EC10	reproduction	2	Deleebeeck et al (2007a)
Simocephalus serrulatus (S)	<2d	17d	EC50	reproduction	2	Deleebeeck et al (2007a)
Simocephalus serrulatus (S)	<2d	17d	LC50	survival	2	Deleebeeck et al (2007a)
Simocephalus vetulus (H)	<2d	21d	EC10	reproduction	2	Deleebeeck et al (2007a)
Simocephalus vetulus (H)	<2d	21d	EC50	reproduction	2	Deleebeeck et al (2007a)
Simocephalus vetulus (H)	<2d	21d	LC50	survival	2	Deleebeeck et al (2007a)

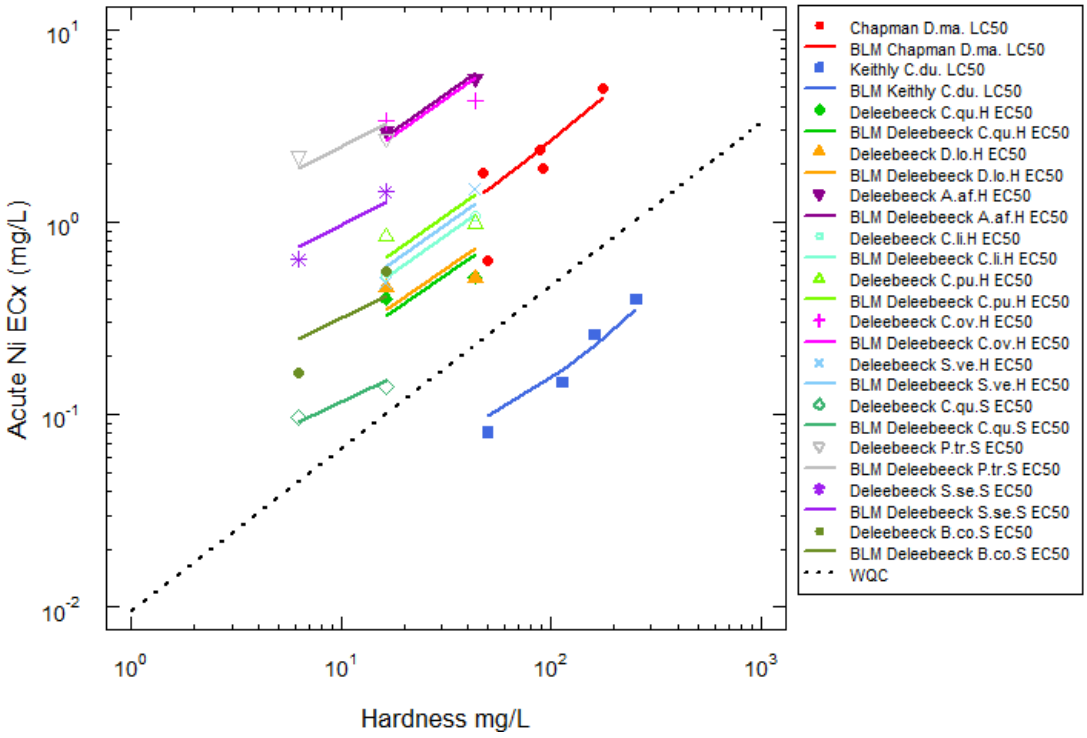

Toxicity Effects showing bioavailability relationships
Ca, Mg
NOM-Ni
Free Ni
BL-Ni
Inorg Ni
Chemical Speciation
Organism Accumulation

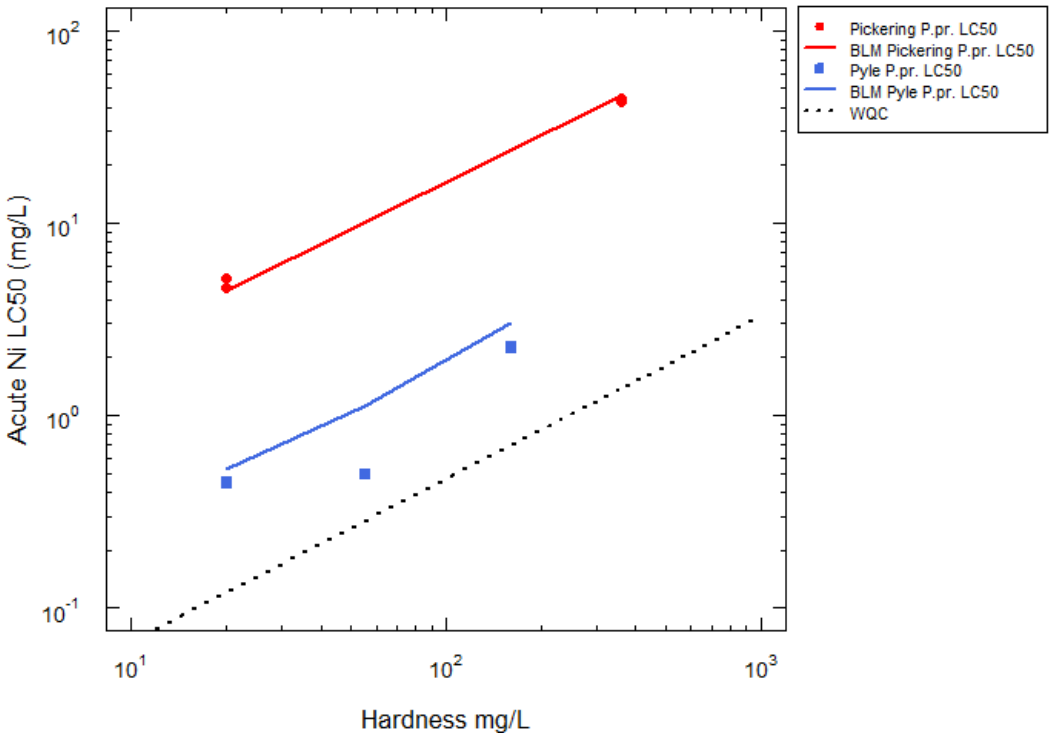
Figure

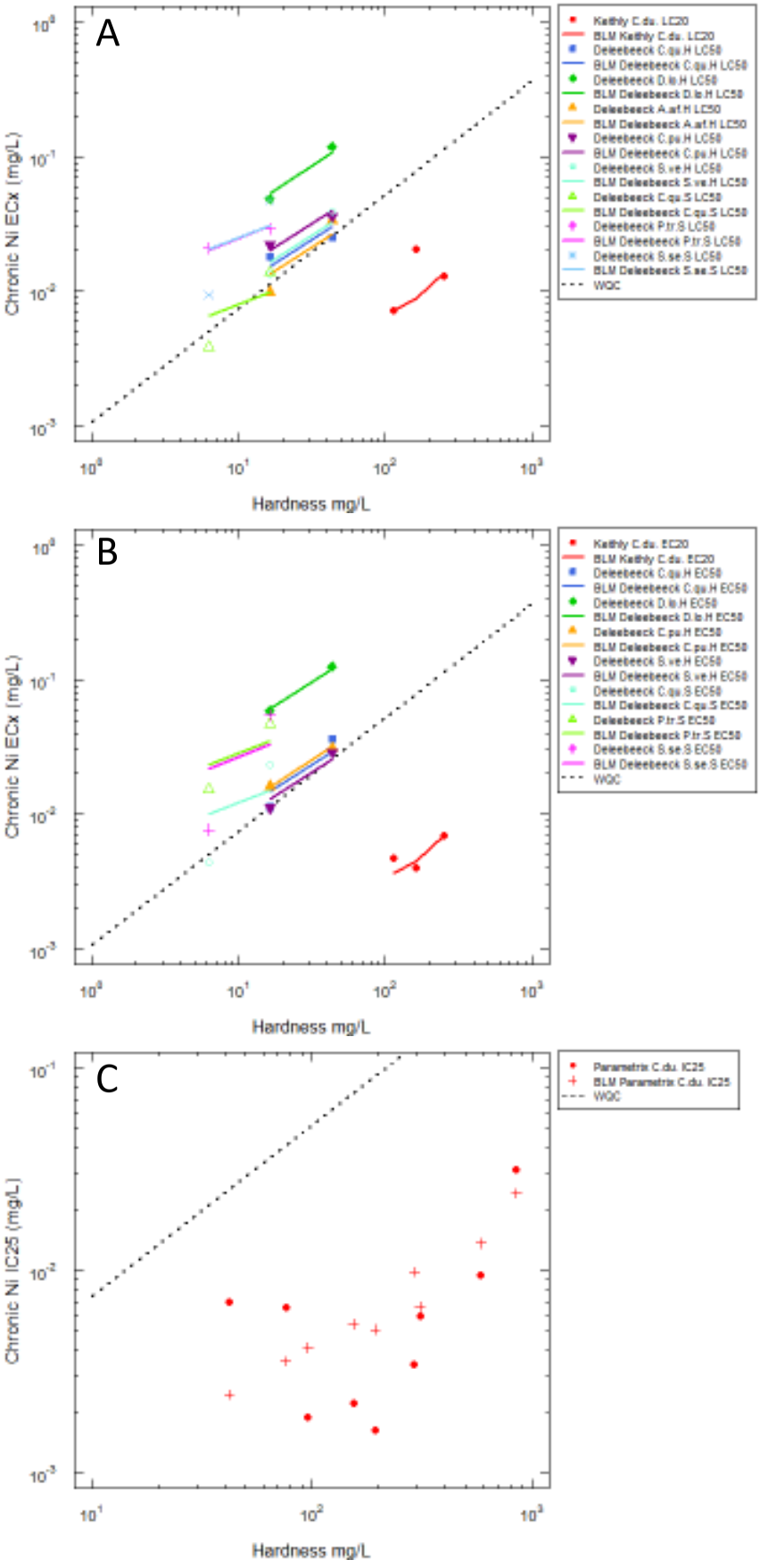

A
Acute Ni ECx / SMEA (mg*gw/L/nmol)
Hardness mg/L
Chapman D.ma. LC50
BLM Chapman D.ma. LC50
Keithly C.du. LC50
BLM Keithly C.du. LC50
Deleebeeck C.qu.H EC50
BLM Deleebeeck C.qu.H EC50
Deleebeeck D.lo.H EC50
BLM Deleebeeck D.lo.H EC50
Deleebeeck A.af.H EC50
BLM Deleebeeck A.af.H EC50
Deleebeeck C.li.H EC50
BLM Deleebeeck C.li.H EC50
Deleebeeck C.pu.H EC50
BLM Deleebeeck C.pu.H EC50
Deleebeeck C.ov.H EC50
BLM Deleebeeck C.ov.H EC50
Deleebeeck S.ve.H EC50
BLM Deleebeeck S.ve.H EC50
Deleebeeck C.qu.S EC50
BLM Deleebeeck C.qu.S EC50
Deleebeeck P.tr.S EC50
BLM Deleebeeck P.tr.S EC50
Deleebeeck S.se.S EC50
BLM Deleebeeck S.se.S EC50
Deleebeeck B.co.S EC50
BLM Deleebeeck B.co.S EC50
B
Acute Ni ECx / SMEA (mg*gw/L/nmol)
Ca mg/L
Deleebeeck D.ma. EC50
BLM Deleebeeck D.ma. EC50
Kozlova D.px. LC50
BLM Kozlova D.px. LC50


A
Acute Ni LC50 (mg/L)
Ca mg/L
Meyer Ptr. LC50
BLM Meyer Ptr. LC50
Ca=2.5, Mg=3.6
Ca=3.5, Mg=3.6
Ca=4.25, Mg=3.6
Ca=4.5, Mg=3.6
B
logR2
BL- Ca logK
All Ca series
Deleebeeck O.mykiss
Deleebeeck D.magna
Kozlova D.pulex
Meyer P.promelas
C
logR2
BL- Mg logK
All Mg series
Deleebeeck O.mykiss
Deleebeeck D.magna
Kozlova D.pulex

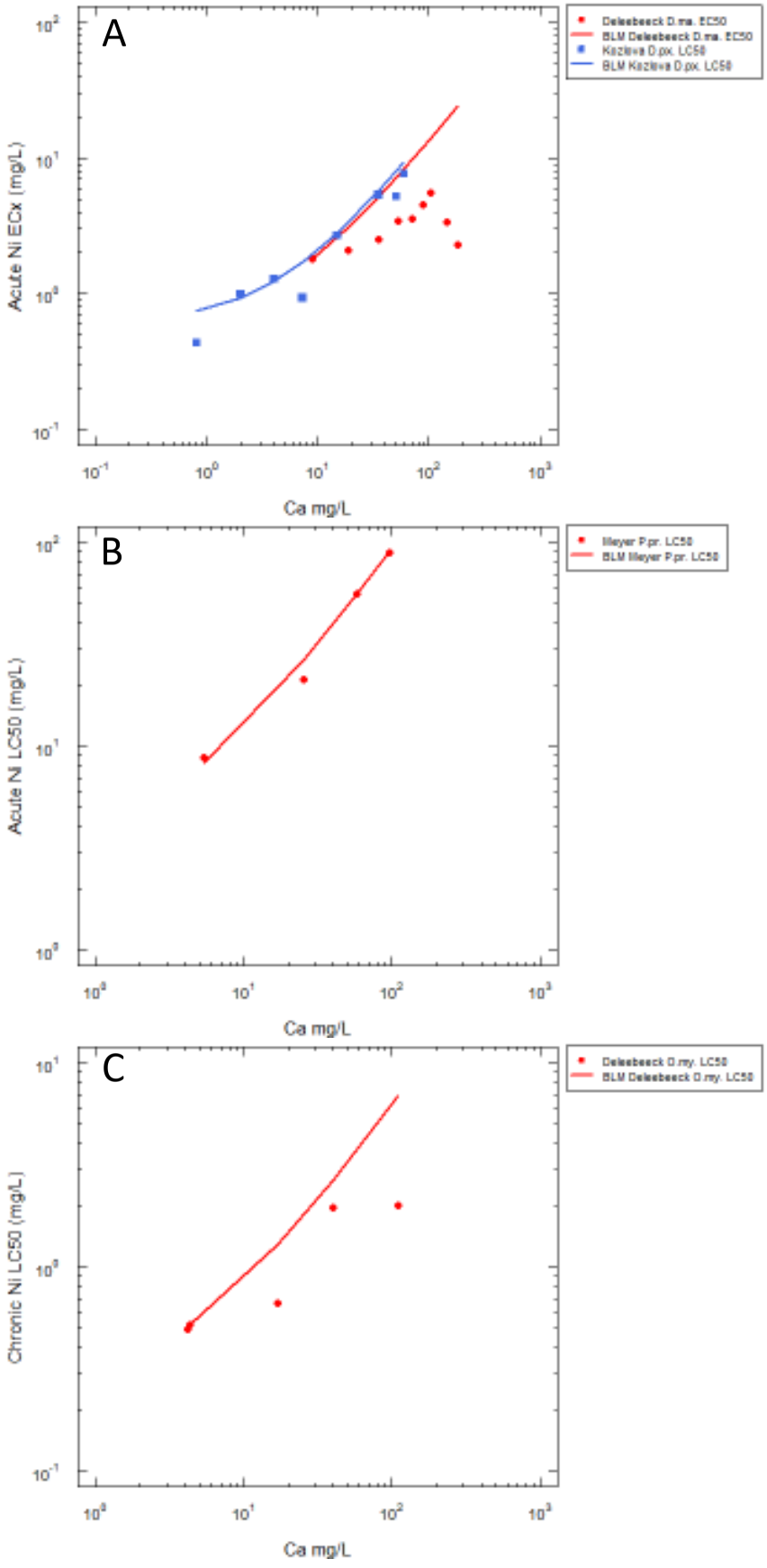
Figure


Acute Ni ECx (mg/L)
Hardness mg/L
Chapman D.ma. LC50
BLM Chapman D.ma. LC50
Keithly C.du. LC50
BLM Keithly C.du. LC50
Deleebeeck C.qu.H EC50
BLM Deleebeeck C.qu.H EC50
Deleebeeck D.lo.H EC50
BLM Deleebeeck D.lo.H EC50
Deleebeeck A.af.H EC50
BLM Deleebeeck A.af.H EC50
Deleebeeck C.li.H EC50
BLM Deleebeeck C.li.H EC50
Deleebeeck C.pu.H EC50
BLM Deleebeeck C.pu.H EC50
Deleebeeck C.ov.H EC50
BLM Deleebeeck C.ov.H EC50
Deleebeeck S.ve.H EC50
BLM Deleebeeck S.ve.H EC50
Deleebeeck C.qu.S EC50
BLM Deleebeeck C.qu.S EC50
Deleebeeck P.tr.S EC50
BLM Deleebeeck P.tr.S EC50
Deleebeeck S.se.S EC50
BLM Deleebeeck S.se.S EC50
Deleebeeck B.co.S EC50
BLM Deleebeeck B.co.S EC50
WQC

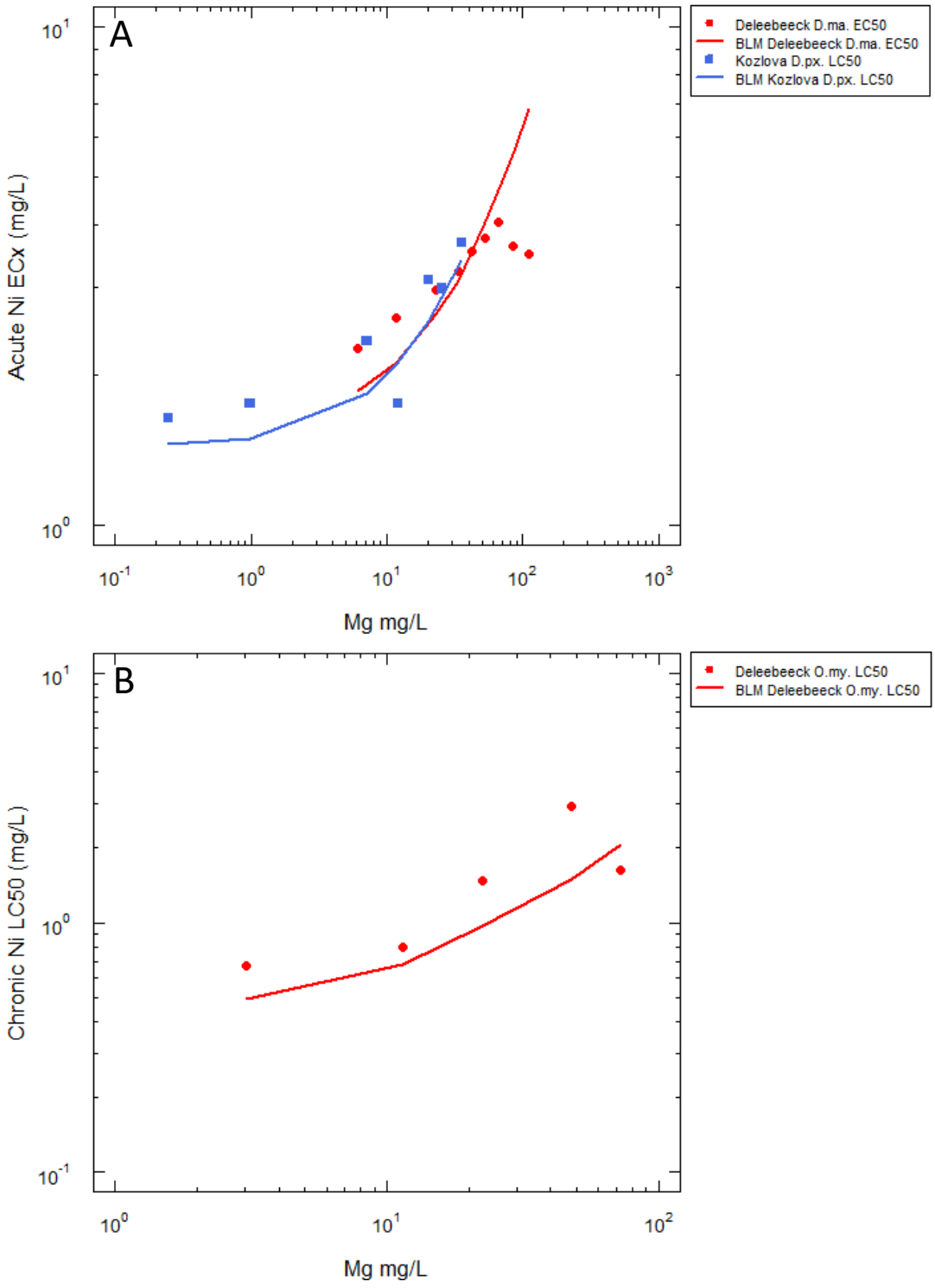

Acute Ni LC50 (mg/L)
Hardness mg/L
Pickering P.pr. LC50
BLM Pickering P.pr. LC50
Pyle P.pr. LC50
BLM Pyle P.pr. LC50
WQC



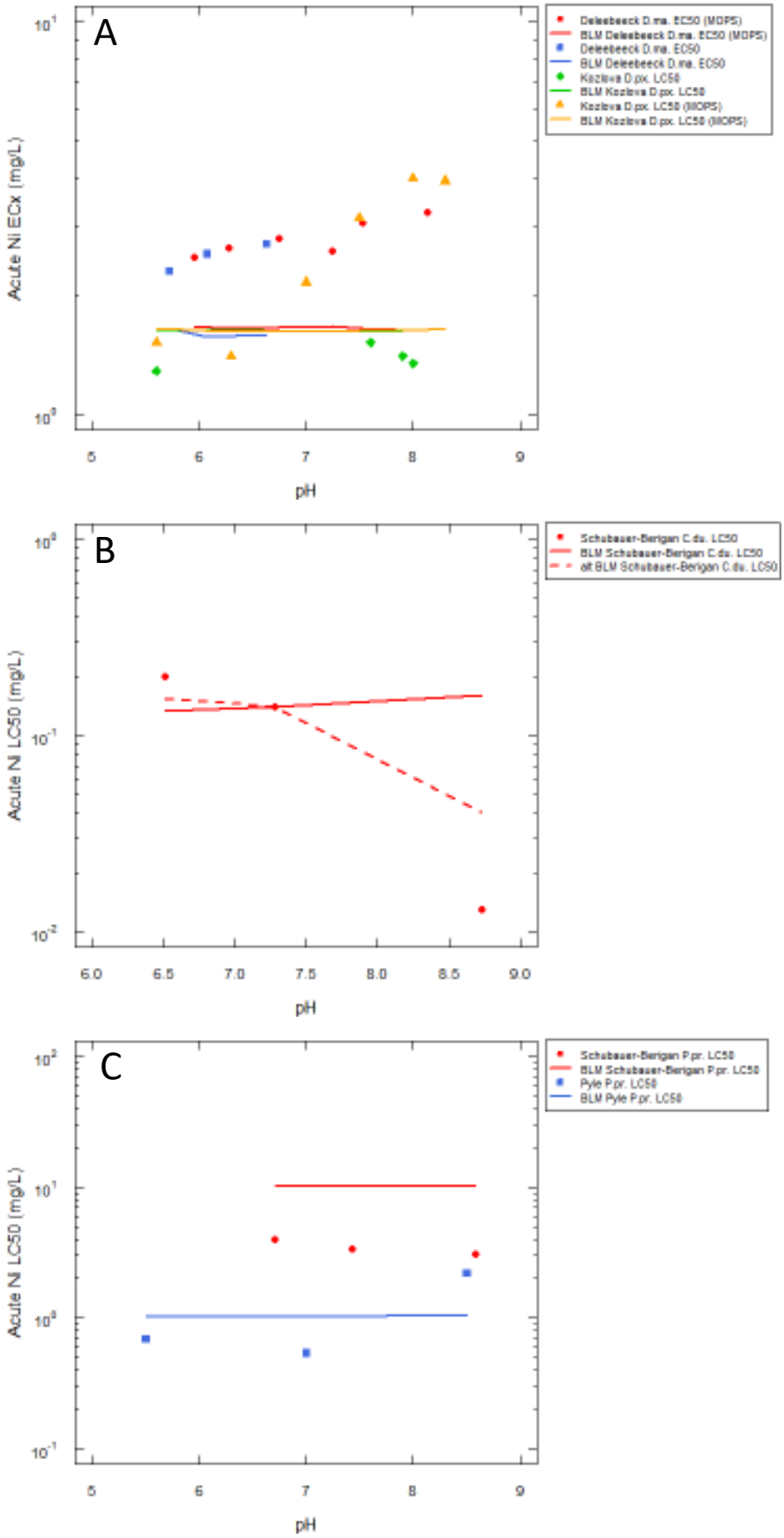
Figure



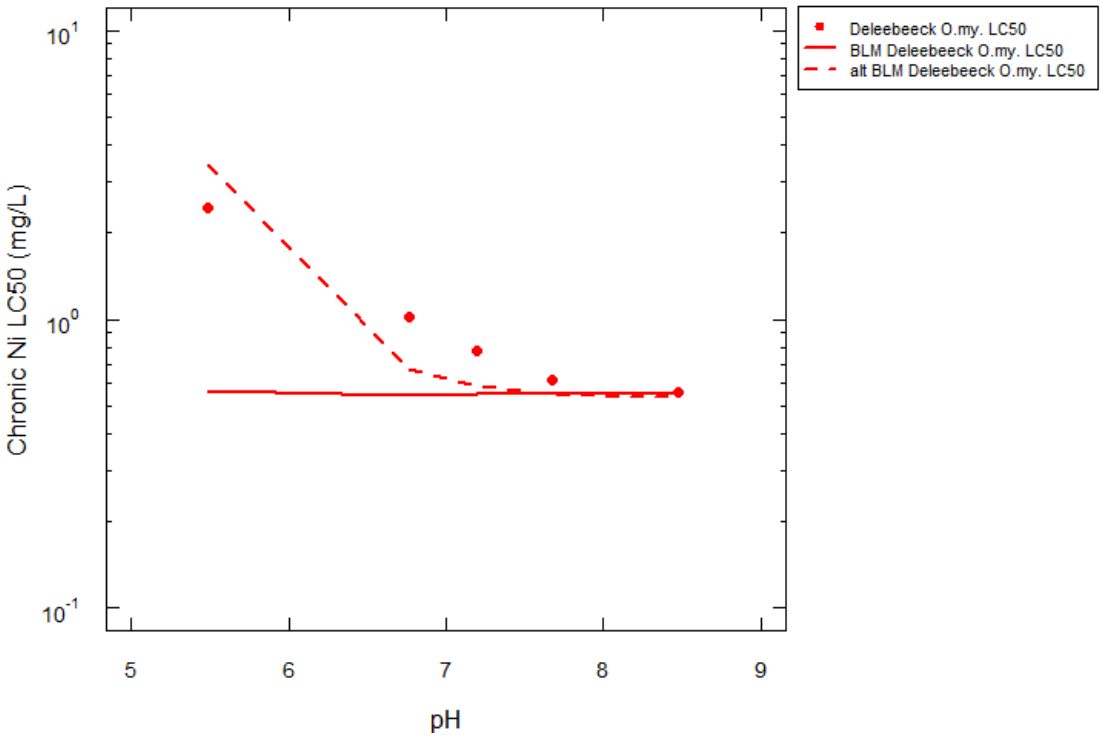
Figure


A
Deleebeeck D.ma. EC50
BLM Deleebeeck D.ma. EC50
Kozlova D.px. LC50
BLM Kozlova D.px. LC50
Acute Ni ECx (mg/L)
Mg mg/L
B
Deleebeeck O.my. LC50
BLM Deleebeeck O.my. LC50
Chronic Ni LC50 (mg/L)
Mg mg/L

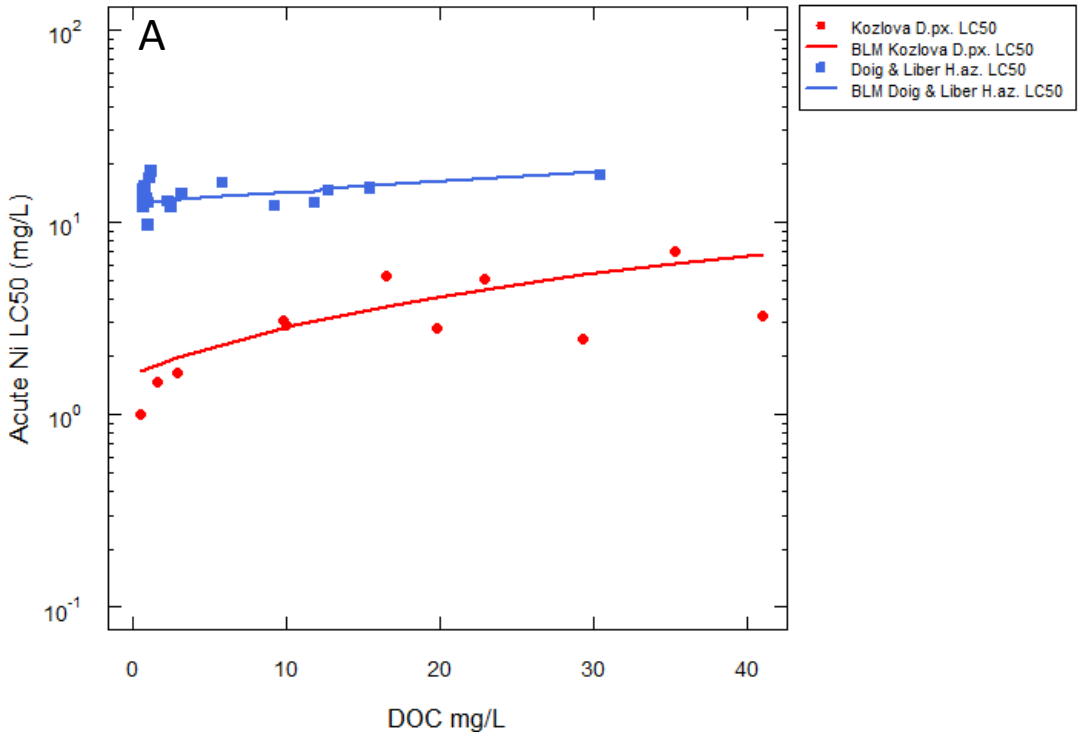
Figure

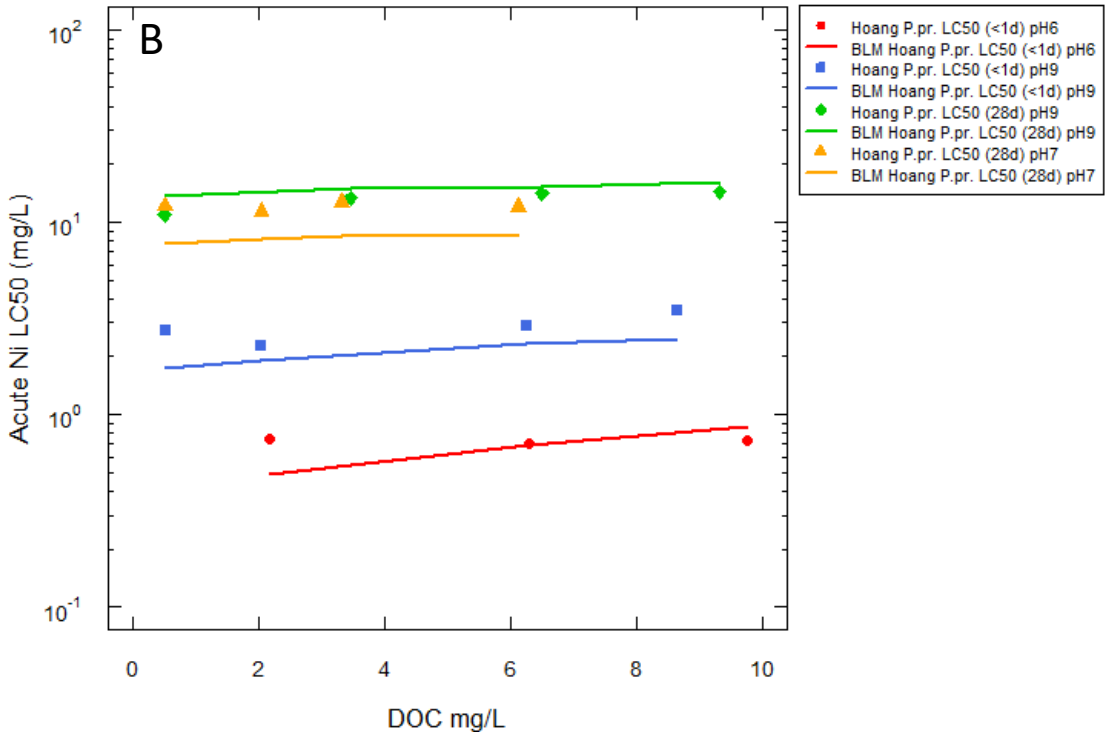

A
Deleebeeck D.ma. EC50 (MOPS)
BLM Deleebeeck D.ma. EC50 (MOPS)
Deleebeeck D.ma. EC50
BLM Deleebeeck D.ma. EC50
Kozlova D.px. LC50
BLM Kozlova D.px. LC50
Kozlova D.px. LC50 (MOPS)
BLM Kozlova D.px. LC50 (MOPS)
Acute Ni ECx (mg/L)
pH
B
Schubauer-Berigan C.du. LC50
BLM Schubauer-Berigan C.du. LC50
alt BLM Schubauer-Berigan C.du. LC50
Acute Ni LC50 (mg/L)
pH
C
Schubauer-Berigan P.pr. LC50
BLM Schubauer-Berigan P.pr. LC50
Pyle P.pr. LC50
BLM Pyle P.pr. LC50
Acute Ni LC50 (mg/L)
pH

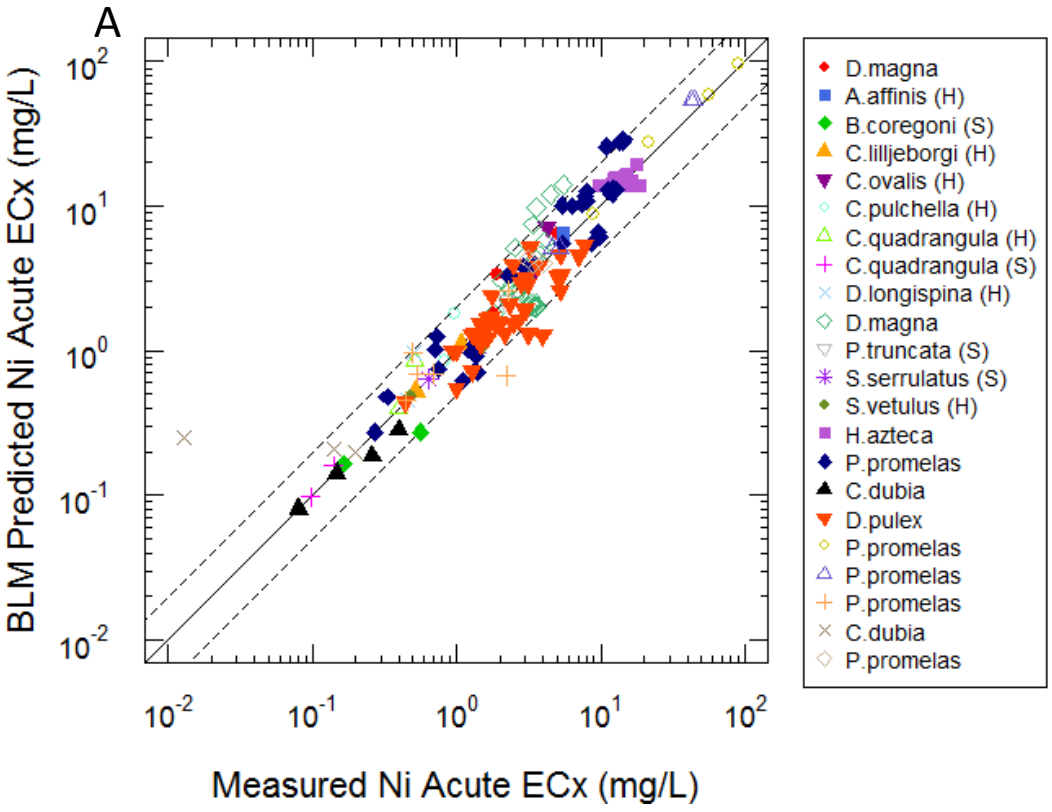
Figure

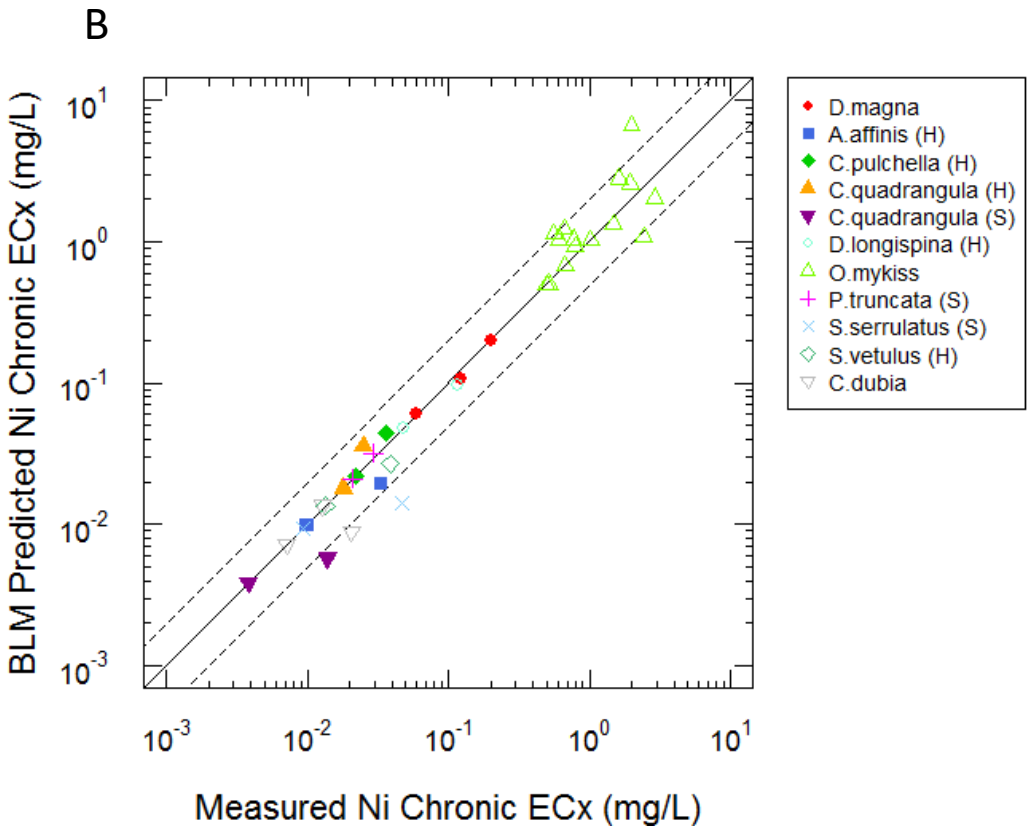

Deleebeeck O.my. LC50
BLM Deleebeeck O.my. LC50
alt BLM Deleebeeck O.my. LC50
Chronic Ni LC50 (mg/L)
10^1
10^0
10^-1
5
6
7
8
9
pH

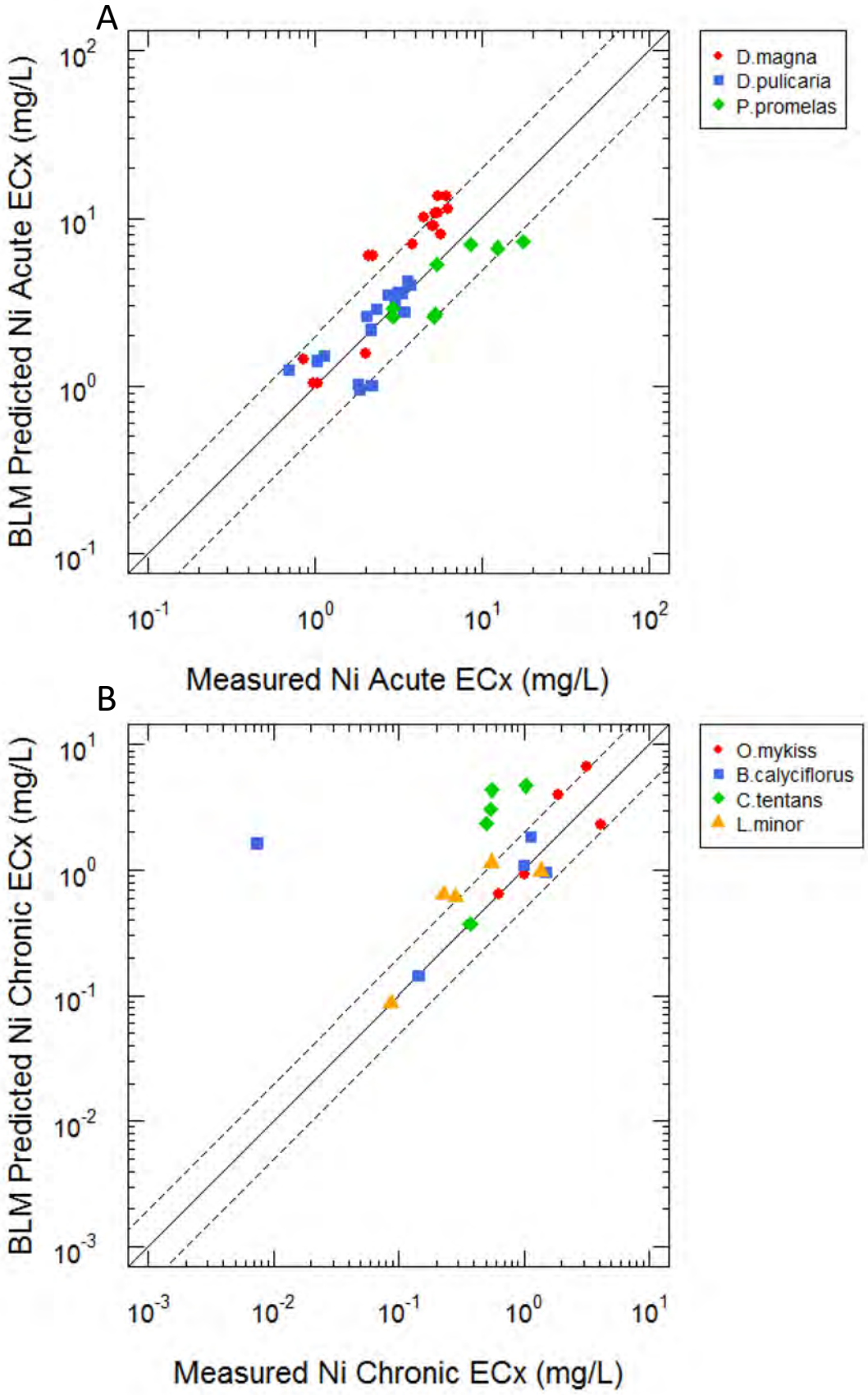
Figure


A
Kozlova D.px. LC50
BLM Kozlova D.px. LC50
Doig & Liber H.az. LC50
BLM Doig & Liber H.az. LC50
Acute Ni LC50 (mg/L)
DOC mg/L


B
Hoang P.pr. LC50 (<1d) pH6
BLM Hoang P.pr. LC50 (<1d) pH6
Hoang P.pr. LC50 (<1d) pH9
BLM Hoang P.pr. LC50 (<1d) pH9
Hoang P.pr. LC50 (28d) pH9
BLM Hoang P.pr. LC50 (28d) pH9
Hoang P.pr. LC50 (28d) pH7
BLM Hoang P.pr. LC50 (28d) pH7
Acute Ni LC50 (mg/L)
DOC mg/L


A
BLM Predicted Ni Acute ECx (mg/L)
Measured Ni Acute ECx (mg/L)
D.magna
A.affinis (H)
B.coregoni (S)
C.lilljeborgi (H)
C.ovalis (H)
C.pulchella (H)
C.quadrangula (H)
C.quadrangula (S)
D.longispina (H)
D.magna
P.truncata (S)
S.serrulatus (S)
S.vetulus (H)
H.azteca
P.promelas
C.dubia
D.pulex
P.promelas
P.promelas
P.promelas
C.dubia
P.promelas


B
BLM Predicted Ni Chronic ECx (mg/L)
Measured Ni Chronic ECx (mg/L)
D.magna
A.affinis (H)
C.pulchella (H)
C.quadrangula (H)
C.quadrangula (S)
D.longispina (H)
O.mykiss
P.truncata (S)
S.serrulatus (S)
S.vetulus (H)
C.dubia


A
BLM Predicted Ni Acute ECx (mg/L)
Measured Ni Acute ECx (mg/L)
D.magna
D.pulicaria
P.promelas
B
BLM Predicted Ni Chronic ECx (mg/L)
Measured Ni Chronic ECx (mg/L)
O.mykiss
B.calyciflorus
C.tentans
L.minor

Figure

Exhibit 27

Aquatic Toxicology Laboratory
33972 Texas St. SW
Albany, Oregon 97321
Office 541-737-2565
Lab 541-926-1254

Exhibit 27

16 March 2016

Tim Kluge

Sanitary District of Decatur
501 Dipper Lane
Decatur, IL 62522

Re: Progress Report – Nickel WER Project

Dear Tim:

We are pleased to submit this progress report for work conducted as part of the Nickel WER project for the Sanitary District of Decatur (SDD).

As part of the effort to determine the potential for ion stress on the test organisms, a "Simulated Effluent" was prepared to match the ionic concentrations of the SDD effluent (based upon water quality parameters measured from historical sampling efforts; Table 1). Acclimation of the test organisms, *Ceriodaphnia dubia*, to this specific ionic content water was employed. During culturing over the course of many months, the organisms appeared fairly healthy, although some reductions in health occurred (observed as reduced reproduction) over this time period. A subsequent discussion occurred with Russ Erickson (USEPA) regarding the preparation of the simulated effluent and the *C. dubia* acclimation and health. A speculation, in terms of organism health during acclimation, was that *C. dubia* may be at a "tipping point" or "limit" of health due to ionic content of the water. With this information and during a later conference call, Russ indicated that he would not object to a "modest" dilution of the effluent (defined as 20-30% maximum) to eliminate potential ion toxicity as a confounding factor. With this information, *C. dubia* were then cultured at a 20% dilution of the Simulated Effluent shown in Table 1.

On December 01, 2015, OSU AquaTox received a 20-Liter sample of SDD Effluent (consisting of a 24 hour composite sample collected from 11/29/15 to 11/30/15). The sample was received in good condition and within the acceptable temperature (0-6°C) and holding period ($\leq$ 36 hrs). The health of the test organism, *Ceriodaphnia dubia*, in the acclimation water (simulated effluent prepared to match the ionic makeup of typical SDD effluent and diluted by 20%) was good and neonates from these cultures were used in toxicity testing. The cultures were acclimated in the diluted Simulated Effluent with success for over a month. For the present toxicity testing and in order to provide a more accurate match of this specific sample of SDD effluent to the simulated reconstituted water (RW) lab effluent, the total dissolved solids (TDS) concentration of the SDD Effluent was measured upon arrival and the SDD effluent was subsequently diluted using deionized water to match the TDS of the Simulated RW Effluent. The SDD effluent was diluted by 20% with deionized water to achieve this match. This 20% dilution was also the percentage employed from previously tested "full strength" Simulated RW which had resulted in mixed effects on the health of the acclimating *C. dubia* culture.

Two short-term chronic toxicity tests (7-day static-renewal tests assessing survival and reproduction [USEPA 2002]) were performed using the 20% diluted SDD Effluent and the Simulated RW Effluent as control/dilution waters in their respective tests. The undiluted SDD

effluent was also tested as a concurrent control water. Water quality characteristics of the waters used in the toxicity tests is reported in Table 2. As shown, the laboratory water and the effluent matched nicely in their compositions with the exception of dissolved organic carbon (DOC). Table 3 reports the priority pollutants (Ag, As, Be, Cd, Cr, Cu, Pb, Hg, Ni, Se, Ag, Th, Zn) measured in the 20% SDD Effluent. None of the values exceed any ambient water quality criteria, if applicable.

Table 1. Historical water quality data: Simulated Effluent compared to SDD Effluent

Water	Hard.	Alk.	Cond.	Ca	Mg	Na	K	Cl^-	SO_4	DOC	pH initial	pH final
	(mg/L as $CaCO_3$)		µS/cm	mg/L							SU	
Simulated Effluent	400	564	2,820	64.0	55.2	446	101	369	350	0.68	8.4	8.6
SDD Effluent (19.2 µg/L Ni)	424	632	3,420	64.1	64.3	557	76.7	464	333	22.4	8.0	8.8

SU = Standard Units. pH is shown as measured on Day 0 and Day 7 of the study.

Table 2. Toxicity testing water quality: Simulated Effluent compared to SDD Effluent

Water	Hard.	Alk.	Cond.	TDS	Ca	Mg	Na	K	Cl^-	SO_4	DOC	pH initial	pH final
	(mg/L as $CaCO_3$)		µS/cm	mg/L								SU	
RW #1304 – 20% diluted simulated effluent	324	436	2,250	1,134	52.5	45.9	348	81.3	315	295	< 0.20	8.6	8.9
SDD Effluent 20% Diluted [A]	276	484	2,420	1,221	39.3	49.0	416	62.1	320	324	11.8	8.4	8.9
SDD Effluent [A]	332	564	2,930	1,501	50.9	59.1	512	77.1	400	399	15.3	8.3	9.0

SU = Standard Units. pH is shown as measured on Day 0 and Day 7 of the study.
A Effluent was filtered through 180 µm mesh prior to use in the toxicity test to remove larger particulates which may hinder biological observations of reproduction.

Table 3. 20% Diluted SDD Effluent (control/dilution water in toxicity test)

Parameter	
Antimony (µg/L) :	0.39
Arsenic (µg/L) :	2.47
Beryllium (µg/L) :	BMDL < 0.025
Cadmium (µg/L) :	BMDL < 0.030
Chromium (µg/L) :	0.85
Copper (µg/L) :	2.80
Lead (µg/L) :	0.43
Mercury (µg/L) :	BMDL < 0.045
Nickel (µg/L) :	19.2
Selenium (µg/L) :	4.39
Silver (µg/L) :	BMDL < 0.025
Thallium (µg/L) :	BMDL < 0.025
Zinc (µg/L) :	8.84

The toxicity test with lab water was a short-term chronic toxicity test using the Simulated Effluent (20% diluted) as the control/dilution water. Exposures were prepared by spiking the control/dilution water with varying concentrations of Ni stock (as $NiCl_2$ x $6H_2O$), allowing a 2-hr equilibrium period, followed by test initiation or daily renewal. In addition to the Simulated Effluent, a concurrent control exposure of very hard reconstituted water was tested. Survival and reproduction were assessed and are reported in Table 4. All concentrations of Ni in the tables below are nominal. There was no statistically significant difference between the very hard reconstituted lab water and the Simulated Effluent (20% diluted). Survival and reproduction in the Ni exposures were compared to those in the Simulated Effluent (20% diluted) control. For survival, the no-observable effect concentration (NOEC) was 14.3 µg/L Ni and a lowest-observable effect concentration (LOEC) was 20.4 µg/L Ni. The median-lethal concentration (LC_{50}) was 20.4 (No 95% CI) µg/L Ni. The 20% lethal effect concentration (LC_{20}) was 14.55 (13.59 – 15.58) µg/L Ni.

**Table 4. Short-term chronic toxicity test –
20% Diluted Simulated Effluent (Laboratory Water)**

Nominal Concentration (µg/L Ni)	Survival (%) ± SD	Reproduction ± SD
Concurrent Control: Very Hard RW	100 ± 0	20.6 ± 7.4
Dilution H_2O Control: 20% Diluted Simulated Effluent	100 ± 0	24.4 ± 3.1
4.9	100 ± 0	22.3 ± 1.4
7	100 ± 0	19.9 ± 4.1
10	100 ± 0	18.3 ± 3.8 *
14.3	80 ± 42.2	12.9 ± 5.5 *
20.4	50 ± 52.7 *	N/A **

* Statistically significant difference ($p \leq 0.05$) from dilution water control (20% diluted simulated effluent).

** Survival effect was observed in this concentration, therefore this concentration was removed from comparison testing for reproduction.

N/A = Not applicable

The toxicity test with effluent was a short-term chronic toxicity test using the SDD Effluent (20% diluted) as the control/dilution water. Exposures were prepared by spiking the control/dilution water with varying concentrations of Ni stock (as $NiCl_2$ x $6H_2O$), allowing a 2-hr equilibrium period, followed by test initiation or daily renewal. Based upon measurements provided by SDD, the undiluted effluent sample provided to OSU contained a Ni concentration of 21.3 µg/L. Therefore spiked concentrations are reported in Table 5 as "+" 19.2 (estimated after the SDD Effluent had been diluted by 20%). An undiluted SDD effluent was included as an exposure. Survival and reproduction were assessed and are reported in Table 5. All concentrations of Ni in the tables below are nominal. The diluted SDD effluent showed toxicity both alone and in all the Ni exposures. Interestingly, the undiluted SDD had better survival and reproduction than its diluted counterpart. Because toxicity was observed in all exposures, no dose response or endpoints were able to be calculated.

Table 5. Short-term chronic toxicity test – 20% Diluted SDD Effluent

Nominal Concentration (µg/L Ni) Dilution water plus spiked Ni	Survival (%)	Reproduction ± SD
Concurrent Control: SDD Effluent (no dilution) 21.3 µg/L Ni	100 ± 0	12.2 ± 3.2
Control/Dilution H_2O: 20% Diluted SDD Effluent 19.2 µg/L Ni	50 ± 52.7	7.6 ± 7.0
19.2 + 7 = 26.2	40 ± 51.6	4.4 ± 5.1
19.2 + 10 = 29.2	40 ± 51.6	5.7 ± 7.4
19.2 + 14.3 = 33.5	33.3 ± 50	6.2 ± 7.1
19.2 + 20.4 = 39.6	30 ± 48.3	5.7 ± 5.0
19.2 + 29.1 = 48.3	30 ± 48.3	3.1 ± 5.5

In the test with Simulated Effluent (biological reported in Table 4), the Nickel Biotic Ligand Model (BLM), based upon the water quality characteristics reported in Table 2, predicted a LC_{20} and EC_{20} of 13.77 and 7.24 µg/L Ni for survival and reproduction, respectively. This prediction was very close to the actual outcome (based upon nominal Ni concentrations) of a survival LC_{20} of 14.55 µg/L Ni and a reproductive EC_{20} of 7.96 µg/L Ni (Table 6). This demonstrates that the BLM is able to accurately predict toxicity in the simulated effluent testing. The BLM predictions for the SDD effluent (20% diluted) were higher than the concentrations tested, yet toxicity was observed at all exposures (19.2 up to 48.3 µg/L Ni). It should be noted that there is no relationship between Ni spikes and toxicity and therefore it is believed that Ni would not be toxic concentrations observed in the effluent.

Table 6. Nickel Biotic Ligand Model (BLM) Prediction versus Actual

Treatment	Survival LC_{20}	Reproduction EC_{20}
	Nominal (µg/L Ni)	
Simulated Effluent (RW #1304)		
BLM Prediction	13.77	7.24
Toxicity test – *C.dubia*	14.55 (13.59 – 15.58)	7.96 (5.55 – 11.42)
SDD Effluent (20% Diluted)		
BLM Prediction	69.14	38.7
SDD Effluent		
BLM Prediction	98.35	55.30

The acclimation of the test organism, *C. dubia*, was an important step for toxicity testing in the high pH, high ionic composition water. In addition, the BLM very accurately predicted effect concentrations, ensuring that this model is appropriate for the determination of site specific criteria for Ni for SDD. Although survival and reproductive effects were observed in the diluted SDD Effluent and accompanying exposures, it appears that toxicity may be occurring due to something else besides ionic composition of the water or Ni. Based upon the status to date, below are some possible next steps.

Possible Next Steps

- Measure Ni concentrations in all test exposures and submit data.
- Submit results as preliminary without measured Ni.
- Additional toxicity tests, if appropriate.

We appreciate the opportunity to work with SDD on this project. If you have any questions or comments, please feel free to give us a call at +1-541-926-1254.

Sincerely,

Allison Cardwell
Sr. Faculty Research Assistant

William Stubblefield, PhD
Professor, Research