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Pseudokirchneriella Subcapitata, Ceriodaphnia Dubia and Rainbow Trout Responses to Uranium Exposure in Combination with Metals, Nutrients and Total Dissolved Solids Mixtures Using Site Water Collected from Two Creeks Located in the Yukon

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Abstract

This study evaluates the effects of uranium (U) in combination with metals (arsenic, copper, chromium and zinc), nutrients (ammonia, sulphate) and total dissolved solids (TDS) on *Pseudokirchneriella subcapitata, Ceriodaphnia dubia* and rainbow trout (*Oncorhynchus mykiss*). Water was collected from two creeks in the Yukon and spiked with a mixture of U, metals, nutrients and TDS. Water was collected at two times of the year (summer and winter). Tests included: a - Metal Mixture, b - Metal Mixture + Nutrients and c - Metal Mixture + Nutrients + TDS with three treatment levels representing low, moderate and high concentrations per test. No adverse effects were noted in the algae after exposure to any of the tests and treatments (i.e., low, moderate and high). A stimulatory effect was observed in the algae which may have implications for enrichment of aquatic environments downstream mines.

For *C. dubia*, no effects occurred for the a - Metal Mixtures test. However, addition of nutrients to the solutions (i.e., test b) produced reproductive effects in winter water for both creeks. Effects occurred at U concentrations ranging from 100 to 180 µg U/L and mostly due declines in the invertebrate osmotic tolerances. The addition of TDS to the mixture (i.e., test c) augmented the appearance of reproductive effects in *C. dubia* and further supports the hypothesis that adverse effects are related to declines in the invertebrate's osmotic tolerance. For rainbow trout, reductions in fish growth occurred after addition of TDS (i.e., test c) at nominal concentrations of 800 and 1,000 mg TDS/L.

Introduction

Uranium (U) concentrations in Canadian freshwater systems are generally below 1 µg/L (Canadian Council of Ministers of the Environment [CCME] 2011) [1]. However, some areas in Canada have naturally occurring U rich deposits. For example, areas located in the Yukon (YT) are characterized by the presence of uraniferous ore deposits which lead to background stream levels of U significantly higher (>100 µg/L) than the applicable short-term(33µg/L) and long-term (15µg/L) water quality guidelines (WQGs) (CCME 2011) [1]. These mineralized areas hold several operational mines and other planned developments, such as placer mining. Due to the mineralized characteristics of these areas in the YT and the naturally elevated U concentrations generally found in streams, there is potential for adverse impacts to aquatic life from increased U mobility released from mines and mills into the receiving environment. Therefore, it is crucial to understand U behaviour in aquatic systems as well as to determine the levels that could adversely affect aquatic biota in these U-rich areas.

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For aquatic biota relevant to Canadian waters, invertebrates (*Hyalella azteca* and *Ceriodaphnia dubia*) and algae (*Pseudokirchneriella subcapitata*) are generally more sensitive to U exposure than northern temperate fish (e.g., rainbow trout (*Oncorhynchus mykiss*); CCME 2011) [1]. For algae, *P.subcapitata*) reported inhibition concentrations (IC10) for growth ranged from 5.4 to 120 µg U/L Acute toxicity in fish ranged from 1,670 to 59, 000 µg/L (CCME 2011) [1]. Reported acute endpoints (e.g., Lethal Concentration [LC] 50) for invertebrates including crustaceans, worms, hydra, insects, and bivalves exposed to U generally range from 150 to 10,000 µg U/L, (Poston, et al. 1984; Khangarot 1991; Bywater et al. 1991; Hyne et al. 1993a, b; Goulet et al. 2015) [2-6]. However, *Ceriodaphnia dubia* emerged as the most sensitive invertebrate to acute U exposure based on the reported 48h LC 50 of 60-89 µg U/L (low water hardness [< 6 mg/L as $CaCO_3$]) by Pickett, et al. (1993) [7]. Long-term exposure to U can also result in chronic effects to aquatic biota. For fish, no effect observed concentrations (NOEC) for U range from 260 to 14, 300 µg/L (CCME 2011) [1]. The reported NOEC for in vertebrates ranged from 1.5 to 2,250 µg U/L (CCME 2011; Muscatello and Liber 2009; Burnett-Seidel and Liber 2006) [1,8,9] with *H. azteca* and *C. dubia* identified as the two most sensitive invertebrate species to long-term aqueous U exposure (CCME 2011) [1].The sensitivities of these two aquatic invertebrates to U exposure generally overlaps and is dictated mainly by differences in water characteristics, a factor that have the capacity to affect metal toxicity, including U (Poston, et al. 1984; Riethmuller et al. 2001; Markich 2002; van Dam et al. 2012) [2,10-12].

Uranium is known to form a variety of complexes with inorganic ligands (e.g., uranyl carbonate, sulphate and/or phosphate), humic and fulvic acids in the form of dissolved organic carbon (DOC), clay and silt particles, iron and manganese oxides (Trenfield, et al. 2011; Crawford et al. 2016) [13]. For example, recently Muscatello, et al. (2020) [14] evaluated the toxicity of U to *C. dubia* exposed to water collected from two creeks in the YT spiked with U and reported chronic endpoints significantly above the CCME water quality guideline (WQG) of 15 µg U/L (no observed effect concentration [NOEC] = 381 µg U/L; lowest observed effect concentration [LOEC] 524 µg U/L). Concentrations of DOC in these creeks during the ice-free period can exceed 10 mg/L and it was postulated that the DOC concentrations present in the collected site water reduced the availability of U and hence, ameliorated its toxicity to *C. dubia*. Dissolved organic carbon is known to complex metals, including U in aquatic systems and the presence of DOC has been demonstrated to reduce U toxicity to freshwater organisms (Trenfield, et. al. 2011) [13]. In addition, U solubility is also affected by pH, alkalinity and to lesser degrees, by hardness (Riethmuller, et al. 2001; Goulet et al. 2015) [10,6]. The presence of other metals in solution is known to affect toxicity responses by either antagonistic, or synergistic processes. However, limited data are available on U interactions with other metals, regarding its toxicity to aquatic organisms. Markich (2002) [11], reported a synergistic effect between U and manganese on the valve movement of the tropical freshwater bivalve, *Velesunio angasi.* Charles, et al. (2006) [15] reported an antagonistic toxic effect between copper (Cu) and U in the tropical duckweed, *Lemna aequinoctialis*. Furthermore, data on the interactions of U and other elements, such as sulphate, ammonia and total dissolved solids (TDS), known to generally occur in natural aquatic environments and in mine discharges, is lacking. All these factors have the potential to confound the responses of aquatic biota to U exposure; therefore, characterizing site-specific factors in natural waters (e.g., DOC, sulphate, ammonia, presence of other metals) and understanding U dynamics in aquatic systems is key to establishing protective water quality guidelines and objectives for U downstream operational mines.

The main objective of this research is to evaluate the responses of a common freshwater algae (*P. subcapitata*), the water flea *C. dubia* and rainbow trout exposed to U in combination with elements commonly found in mine discharges, including metals (arsenic [As], chromium [Cr], Cu and zinc [Zn]), nutrients (ammonia and sulphate) and TDS, using spiked site-water collected from two un-impacted streams in a proposed gold mine development area in the YT. The algae, invertebrate and fish species selected for these tests are good candidates for assessing the aqueous toxicity of U since as previously mentioned, *P. subcapitata* and *C. dubia* are known to be sensitive aquatic organisms to U exposure and all three species are known to be widely distributed in Canadian waters.

Material and Methods

Site water collection

Toxicity tests were performed using site water collected from Latte Creek and Halfway Creek, located in the White Gold District of west-central YT, approximately 130 km south of Dawson City (Figure 1). The creeks are predominantly erosional with rocky, cobble and boulder substrates and limited pool areas. Flows in this area tend to be seasonal, and mostly driven by spring freshet (high-flow) in surface water and groundwater sources at winter times (low-flow). Complete winter ice cover generally occurs in the upper creeks, with *aufeis* observed in some areas of the creeks. During winter low-flow periods and influenced mostly by groundwater input, U concentrations in these creeks have consistently been above water quality guidelines for the protection of aquatic life (15 µg/L [long-term], CCME [2011] [1]).

Site water was collected from Latte and Halfway Creeks on March 17, 2018 and August 20-21, 2018.Water was collected during open-water and ice-covered water to represent characteristics of seasonal flows (summer-high and winter-low flows, respectively). Samples were delivered to the toxicological laboratory (where toxicity tests were performed) within 72-hrs after collection (recommended holding time by Environment Canada 2007a, b) [16,17]. Samples were transported in 20-L plastic containers and inside coolers containing freezer packs to keep temperature during transport at approximately 4 °C. After arrival, samples were stored in the dark at 4 (± 2) °C until use in the tests. Toxicity testing commenced within 48-hrs after water arrival. A subsample of collected site water along with a sample of laboratory water were sent for general chemistry

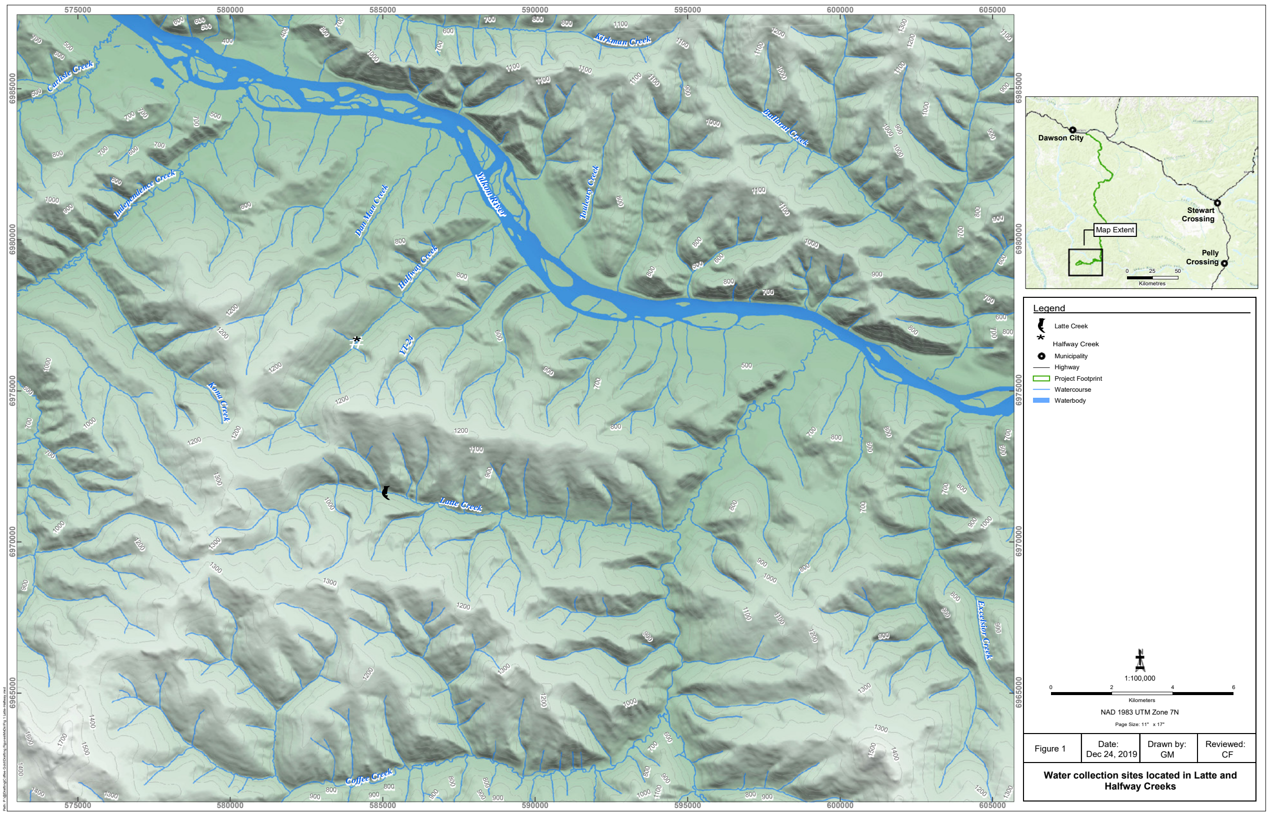

Figure 1

determinations (e.g., total suspended solids [TSS]) and total and dissolved metals analysis after arrival to the laboratory facilities (Figure 1).

Toxicity Tests

Three aquatic species were used in the toxicity tests, algae (*P. subcapitata*), the invertebrate *C. dubia* and rainbow trout all of which are widely used in laboratory tests and considered sensitive to U exposure. Test species were also chosen on the basis of providing a suitable representation of aquatic biota present in Latte Creek and Halfway Creek. All organisms were obtained from the same population and sourced from well established laboratory colonies at Nautilus Environmental Company (Nautilus); Burnaby, BC. Toxicity tests included a) singly exposure to Metal Mixtures (As, Cu, Cr, and Zn), b) Metal Mixtures + Nutrients (sulphate and ammonia) and c) Metal Mixtures + Nutrients + TDS (Table 1) to evaluate the potential for interactions between these elements and U. Exposure solutions were prepared using analytical grade chemical (SCP Science [Quebec, Canada]) diluted with site water collected from Latte Creek and Halfway Creek during winter and summer flows periods (with the exception of test c, which was only performed using summer collected water) (Figure 1; Table 1). Test concentrations for U were based on calculated 95th percentile background values known to occur in the creeks (31 µg U/L and 86 µg U/L for Latte and Halfway Creek, respectively [Muscatello, et al. 2020]) [14]. Uranium was added to these mixtures in incremental concentrations to achieve 3 test solutions: Low (to include the lower background value of 31 µg U/L), moderate (to include the highest background value 86 µg U/L) and high (to represent concentrations generally above background). Selection of additional metals (e.g., As, Cu, Cr and Zn) for inclusion in the test solutions were based on professional experience of elements of concern and concentrations with the potential to occur downstream of operational mines in the YT (Table 1). In addition, nutrients, such as sulphate and ammonia were added to the test solutions as these two elements are known to occur in mining operations as a result of operational/treatment activities and explosives use in blasting, respectively.

Experimental Procedures

A summary of the test conditions is shown in (Table S1). Exposures were conducted in an environmental chamber with a set photoperiod and controlled temperature. *P. subcapitata* tests were performed with 3-7 day old logarithm growth phase algae in micro plates. The duration of tests was 72-hrs, static and followed protocols described in Environment Canada (2007b) for algae exposures. For *C. dubia*, tests were started with < 24-hrs old neonates; test duration was 7-days and followed protocols described by Environment Canada (Environment Canada [2007a]) [16]. Exposure of organisms to test solutions was conducted in 20-ml glass tubes following a static renewal protocol. Test organisms were fed daily using a 3:1 ratio of *P. subcapitata* cells and yeast-cerophyl-trout chow mix (YCT; Aquatic Biosystems [ABS], Fort Collins, CO). Rainbow trout tests were performed with 2-6 days post-swim-up fry as required by established protocols (Lazorchak and Smith 2007) [18]. Tests were conducted in 1-L glass

Table 1: Metal, nutrients and total dissolved solids (TDS) shown as nominal concentrations for the low, moderate and high exposure solutions generated using site water collected from Latte and Coffee Creeks at two times of the year.

Test Type	**a) Metal Mixtures**							
	b) Metal Mixtures + Nutrients							
	c) Metal Mixtures + Nutrients + TDS*							
Mixture Solution	**Copper** (µg/L)	**Chromium** (µg/L)	**Arsenic** (µg/L)	**Uranium** (µg/L)	**Zinc** (µg/L)	**Sulphate** (mg/L)	**Ammoni a** (mg/L; as N)	**TDS** (mg/L)
Chemical Form[1]	Copper Chloride Dihydrate	Ammonium Dichromate	Potassium Dihydrogen Arsenate	Uranyl Nitrate Hexahydrate	Zinc Sulphate Heptahydr ate	Calcium Sulfate Dihydrate and Magnesium Sulfate Heptahydrate	Ammonium Chloride	See footnote 2
Low	3.5	1.5	2	31	4	300	2	600
Moderate	3.5	1.5	5	86	8	300	2	800
High	3.5	1.5	8	100	16	300	2	1,000

Notes: 1 - Chemical forms used to prepare mixture solutions; 2 -TDS concentrations were achieved by mixing calcium chloride dihydrate, magnesium chloride hexahydrate, calcium sulfate dehydrate and magnesium sulfate heptahydrate N -Nitrogen; * Test were only performed using water collected in summer from both, Latte and Coffee creeks.

containers following static-renewal protocol. Fish were fed twice daily with newly hatched brine shrimp nauplii (*Artemia sp*.) from an *in-house* culture. Aeration was not provided for any of the tests; however dissolved oxygen (DO) levels were recorded daily. Toxicity tests were performed by Nautilus, a certified laboratory by the Canadian Association for Laboratory Accreditation (CALA) and in accordance with applicable protocols (Environment Canada 2007a,b; Lazorchak and Smith 2007) [16-18].

Measurements of mortality, dissolved oxygen (DO), conductivity, pH and temperature were performed daily for the *C.dubia* and rainbow trout tests. Temperature and DO were recorded daily for the static algae test. For static-renewal tests, water was replaced in its totality daily to maintain adequate water quality and adequate exposure concentrations. Samples for determinations of As, Cu, Cr, Zn, ammonia, sulphate and TDS were collected from randomly selected test tubes at test initiation, halfway and finalization prior to water change for the invertebrate and fish tests and at test initiation for the algae test. ALS Laboratories (Burnaby, BC) a CALA certified laboratory performed all elemental analyses.

All testing included a negative (i.e., laboratory control prepared with dechlorinated laboratory water) and positive control (prepared using a reference toxicant; Table S1) to evaluate the quality and reproducibility of the performed test. In addition, site controls prepared with collected site water were used to evaluate potential effects of un-spiked site water on exposed organisms. Evaluated toxicity endpoints included growth inhibition in the algae *P. subcapitata* evaluated using the number of algal cells/mL, survival and reproduction (evaluated as the number of produced broods) in the water flea *C. dubia*, and survival and growth (evaluated as dry weight [d.w]) in rainbow trout. To evaluate fish d.w, rainbow trout were euthanized, and specimens dried for 24-hrs at 60 °C before weight determinations.

Statistical Analysis

Statistical analyses were conducted using Comprehensive Environmental Toxicity Information System (CETIS) statistical software package (Tidepool Scientific Software, 2013) [19] based on measured metal dissolved concentrations and used un-amended site water as the control value. For statistical analyses of toxicological data, significance level was established at $\alpha = 0.05$. Statistical analysis included Fisher Exact Test followed by Bonferroni-Holm comparisons for analysis of survival data, and analysis of the variance (ANOVA) on Ranks followed by Dunnett's multiple comparisons for analysis of reproduction data.

Results

Site water characterization

General water quality parameters for controls including laboratory and un-amended Latte and Halfway Creeks waters are summarized in the Supplemental Material (Tables S2 to Table S3). The main noticeable differences in site waters between the two evaluated seasonal flow periods were the concentration of U, DOC, conductivity and hardness. Latte Creek water is generally characterized by low concentrations of TSS (< 3 mg/L), hard water and circum neutral pH. Concentrations of U were higher in the winter months along with conductivity and hardness. Uranium concentrations ranged from 8 µg/L and 24 µg/L for summer and winter, respectively. Conductivity and hardness values were approximately two times higher in the winter season (conductivity = 550 µS/cm; hardness = 390 mg/L [as $CaCO_3$]) than those recorded in summer (conductivity = 308 µS/cm; hardness = 151 mg/L [as $CaCO_3$]). Alkalinity was 86 to 92 mg/L (as $CaCO_3$) for summer and winter collected water, respectively. The concentrations of DOC ranged from 4.8 mg/L and 8.2 mg/L for winter and summer, respectively.

For Halfway Creek, water is generally characterized by low concentrations of TSS (< 3 mg/L), hard water and circumneutral pH. Similar to Latte Creek, concentrations of U in Halfway Creek were higher in winter (74 µg/L) than in summer (23. µg/L). Conductivity and hardness values were approximately two times higher in the winter season (conductivity = 340µS/cm; hardness = 300 mg/L [as $CaCO_3$]) than those recorded in summer (conductivity = 195µS/cm; hardness = 88 mg/L [as

$CaCO_3$]). Concentrations of DOC were 4.3 mg/L and 9.4 mg/L for winter and summer periods, respectively. Alkalinity was 66 to 78 mg/L (as $CaCO_3$) for summer and winter collected water, respectively. This creek appears to generally have slightly lower hardness and conductivity values than those recorded for Latte Creek (Supplemental Material Tables S2 and S3).

Test conditions

A summary of experimental conditions including pH, temperature, DO, hardness and conductivity measurements throughout the test is provided in (Tables S4 to S7) of Supplemental Material. Measured DO was consistently >7 mg/L for all tests and values for the other parameters remained consistent for the length of the experiment. Measured As, Cu, Cr, U, Zn, sulphate, ammonia and TDS concentrations in exposure water resembled targeted nominal concentrations (Table S7). With exception of arsenic, all metals exceeded applicable WQGs (CCME 1999) [20] whereas ammonia and sulphate concentrations fell within guideline values. Brief descriptions of exposure water parameters are provided for each test: a - Metal Mixtures, b - Metal Mixtures + Nutrients, and c - Metal Mixtures + Nutrients + TDS in the following sections.

Exposure to the reference toxicant conducted during this testing program fell within the acceptable range for organism performance of mean and two standard deviations in all tests. Mean sensitivity values based on laboratory historical data ranged from 1.8 - 2.0 g Sodium [Na]/L and 0.8 - 1.9 g Na/L for *C. dubia* survival [LC50] and reproduction [inhibitory concentration (IC50)], respectively; 26.0 - 40.2μg Zn/L for *P.subcapitata* growth (IC50) and29.1 - 153.2 μg Cu/L and 28.8 - 148.4μgCu/L for rainbow trout survival (LC50) and growth (IC50), respectively. Thus, the sensitivity of the organisms used in these tests is considered appropriate. All laboratory controls passed the test validity requirements listed in (Table S1).

Exposure tests

a) Metal mixtures: General water quality parameters for Latte and Halfway Creeks exposures are summarized in Tables S4 and S7 (Supplemental Material) and briefly described below. Water quality remained constant through the tests and was within acceptability requirements for all evaluated species. Exposure water was generally characterized by circumneutral pH with maxima value of 8.0. Dissolved oxygen levels ranged from 7.7 to 9.8 mg/L throughout the test. Hardness values ranged from 117 to 126mg/L (as $CaCO_3$) and from 314 to 322 mg/L (as $CaCO_3$) for Latte Creek in summer and winter collected water, respectively. For Halfway Creek hardness values were < 100 mg/L (as $CaCO_3$) in the summer and approximately 200 mg/L (as $CaCO_3$) in winter collected water. Conductivity was approximately 250 μS/cm and 580 μS/cm for Latte Creek in summer and winter, respectively. For Halfway Creek conductivity values were160 μS/cm in summer and 400 μS/cm in winter collected water. Generally, Halfway Creek conductivity and hardness values were slightly lower than those recorded in Latte Creek (Tables S4 and S7).

Toxicity test results using metal spiked site water collected from Latte and Halfway Creek during winter and summer periods are summarized in Table 2 to Table 4. For algae, an increase in the number of algal cells that was significantly different ($p < 0.05$) from laboratory control was observed in both Latte and Halfway Creek waters. This stimulatory effect was more noticeable in winter collected water for both creeks. No other significant differences were observed in algal growth between creeks and/or between treatments (i.e., low, moderate, and high) (Table 2).

Generally, no reductions were recorded on the survival and reproduction of *C. dubia* exposed to any of the treatment solutions (Table 3). Although not significant, a slight reduction in reproduction seemed to occur in Latte Creek site water collected during the summer compared to laboratory control. No statistical differences were found in *C. dubia* reproduction between evaluated site waters. For rainbow trout, no adverse effects were observed in survival and/or growth (expressed as d.w) after exposure to low, moderate and high test solutions (Table 4). In addition, significant differences in survival and growth between creeks and seasons were not apparent.

b) Metal Mixtures + Nutrients: General water quality parameters in Latte and Halfway Creeks are summarized in the Supplemental Material (Tables S5 and S7) and briefly described below. Water quality remained constant through the tests and was within acceptability requirements for all evaluated species. Exposure water was generally characterized by circumneutral pH with a maxima value of 8.3. Dissolved oxygen levels ranged from 7.6 to 9.6 mg/L throughout the test (Table S5, Supplemental Material). Concentrations of ammonia in site water were approximately 0.8 mg/L (as nitrogen [N]) and < 0.005 mg/L (as N) for creek waters collected in winter and summer, respectively (Tables S2 and S3, Supplemental Material). Concentrations of sulphate for Latte Creek were 163 mg sulphate/L collected in winter and 71 mg sulphate/L in summer. For Halfway Creek sulphate concentrations were 78 and 29 mg sulphate/L for winter and summer, respectively (Tables S2 and S3, Supplemental Material). Hardness values ranged from 385 to 422 mg $CaCO_3$/Land from 591 to 632 mg $CaCO_3$/L for Latte Creek in collected summer and winter water, respectively (Table S7 Supplemental Material). For Halfway Creek hardness values ranged from 331 to 412 mg$CaCO_3$/L in summer and from 490 to 564 mg$CaCO_3$/L in winter collected water. Conductivity values in Latte and Halfway Creek exposure waters ranged approximately from 700 and 1,000 μS/cm for summer and winter, respectively (Table S7 Supplemental Material). Conductivity values increased in the low, moderate and high exposure treatments relative to site control due to salts and other inorganic chemicals added to achieve targeted exposure concentrations. Hardness and conductivity values are higher in winter than those measured in summer collected water for both creeks.

Results for the Metals Mixtures + Nutrients toxicity test using Latte and Halfway Creeks waters collected during the winter and summer period are summarized in (Tables 5 to 7). A significant ($p < 0.05$) stimulatory effect of site waters compared to laboratory controls was noticeable for algae growth in all treatments, which was consistent with the response recorded in the a - Metal Mixture Test Section

Table 2: Growthmeasurements (calculated as cell yield) for *Pseudokirchneriella subcapitata* exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc generated using spiked Latte and Halfway Creek water collected in winter and summer**.**

Treatments	Winter	Summer
	Cell Yield (x10^4 cells/mL)	Cell Yield (x10^4 cells/mL)
Latte Creek		
Laboratory Control	30.60 ± 2.50	27.60 ± 1.40
Latte Creek (Site Control)	169.40 ± 11.60*	101.50 ± 8.60*
Low	168.30 ± 12.0*	102.50 ± 7.0*
Moderate	159.80 ± 5.30*	99.50 ± 5.80*
High	159.80 ± 1.80*	96.50 ± 6.10*
Halfway Creek		
Laboratory Control	30.80 ± 2.60	27.90 ± 1.50
Halfway Creek (Site Control)	173.80 ± 11.0*	94.90 ± 8.80*
Low	177.80 ± 7.40*	95.80 ± 5.20*
Moderate	183.50 ± 5.80*	97.80 ± 6.80*
High	162.50 ± 6.40*	92.0 ± 6.70*

Notes: * Significantly different ($p < 0.05$) from laboratory control.

Table 3: Survival and reproduction results for *Ceriodaphnia dubia*exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc generated using spiked Latte and Halfway Creek water collected in winter and summer**.**

Treatments	Winter		Summer	
	Survival (%)	Reproduction (Number of broods)	Survival (%)	Reproduction (Number of broods)
Latte Creek				
Laboratory Control	100	21.80 ± 7.60	100	20.50 ± 4.20
Latter Creek (Site Control)	100	20.20 ± 7.50	100	15.20 ± 8.40
Low	100	23.30 ± 1.80	100	19.10 ± 7.60
Moderate	100	21.30 ± 8.0	100	16.50 ± 8.90
High	100	19.80 ± 7.60	100	17.20 ± 10.50
Halfway Creek				
Laboratory Control	100	20.80 ± 9.30	100	22.70 ± 9.50
Halfway Creek (Site Control)	90	23.80 ± 6.0	100	24.80 ± 6.70
Low	100	19.40 ± 10.90	100	21.30 ± 8.20
Moderate	100	21.60 ± 8.30	100	20.70 ± 8.40
High	100	23.30 ± 2.60	100	19.10 ± 8.40

Notes: Calculation of the survival standard deviation was not possible due to the lack of variability in the results.

Table 4: Survival and growth results (mean ± standard deviation) for rainbow trout exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc generated using spiked Latte and Halfway Creek water collected in winter and summer.

Tests Solutions	Winter		Summer	
	Survival (%)	Growth [1] (mg)	Survival (%)	Growth [1] (mg)
Latte Creek				
Laboratory Control	100	40.10 ± 1.20	100	39.0 ± 2.80
Latte Creek (Site Control)	100	41.20 ± 2.50	95 ± 10	41.40 ± 2.30
Low	100	38.10 ± 1.0	100	39.30 ± 2.30
Moderate	100	39.80 ± 3.20	100	39.70 ± 1.50
High	100	37.90 ± 1.50	100	40.60 ± 1.20
Halfway Creek				
Laboratory Control	100	40.10 ± 1.20	95 ± 10	42.10 ± 1.60
Halfway Creek (Site Control)	100	40.0 ± 3.10	95 ± 10	41.0 ± 1.60
Low	100	38.50 ± 2.20	100	37.90 ± 1.90
Moderate	100	39.40 ± 2.90	100	40.40 ± 1.10
High	100	38.60 ± 1.60	85 ± 19.20	37.90 ± 3.20

Notes: 1 -Expressed as mg of dry weight**;** * Significantly different ($p < 0.05$) from laboratory control; Calculation of the survival standard deviation was not possible for some of the treatments due to the lack of variability in the results.

Table 5: Growth measurements (calculated as cell yield) for *Pseudokirchneriella subcapitata* exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc + nutrients (sulphate and ammonia) generated using spiked Latte and Halfway Creek water collected in winter and summer.

Treatments	Winter	Summer
	Cell Yield (x10^4 cells/mL)	Cell Yield (x10^4 cells/mL)
Latte Creek		
Laboratory Control	31.50 ± 2.80	27.40 ± 1.70
Latte Creek (Site Control)	169.40 ± 11.60*	79.90 ± 3.30*
Low	143.80 ± 8.50*#	76.0 ± 3.80*
Moderate	177.50 ± 7.90*	75.20 ± 3.50*
High	176.30 ± 6.60*	75.20 ± 4.30*
Halfway Creek		
Laboratory Control	30.10 ± 2.30	26.60 ± 1.70
Halfway Creek (Site Control)	173.80 ± 11*	72.80 ± 4.70*
Low	167.50 ± 8.90*	72.0 ± 2.90*
Moderate	173.0 ± 8.0*	75.0 ± 3.70*
High	189.50 ± 6.0*	71.80 ± 5.0*

Notes: Data represent mean ±standard deviation; * Significantly different ($p < 0.05$) from laboratory control; # Significantly lower ($p < 0.05$) than site control.

Table 6: Survival and reproduction results (mean ± standard deviation) for *Ceriodaphnia dubia* exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc + nutrients (sulphate and ammonia) generated using spiked Latte and Halfway Creek water collected in winter and summer.

Treatments	Winter		Summer	
	Survival (%)	Reproduction (Number of broods)	Survival (%)	Reproduction (Number of broods)
Latte Creek				
Laboratory Control	100	21.80 ± 7.60	100	17.50 ± 1.30
Latter Creek (Site Control)	100	20.20 ± 7.50	100	23.10 ± 3.80
Low	100	22.10 ± 6.20	100	23.30 ± 2.80
Moderate	100	15.20 ± 5.60*#	100	22.20 ± 2.0
High	100	18.20 ± 8.50	100	20.10 ± 2.60
Halfway Creek				
Laboratory Control	100	20.60 ± 9.30	100	18.10 ± 3.20
Halfway Creek (Site Control)	90	23.80 ± 6.0	100	17.60 ± 7.80
Low	100	24.50 ± 4.40	100	18.40 ± 6.0
Moderate	100	20.30 ± 8.60	100	18.70 ± 4.90
High	90	16.50 ± 7.90*#	100	18.40 ± 4.30

Notes: * Significantly different ($p < 0.05$) from laboratory control; # Significantly different ($p < 0.05$) from site control; Calculation of the survival standard deviation was not possible due to the lack of variability in the result

Table 7: Survival and growth results (mean ± standard deviation) for rainbow trout exposed to low, moderate and high mixtures of arsenic, copper, chromium, uranium and zinc + nutrients (sulphate and ammonia) generated using spiked Latte and Halfway Creek water collected in winter and summer**.**

Treatments	Winter		Summer	
	Survival (%)	Growth[1] (mg)	Survival (%)	Growth[1] (mg)
Latte Creek				
Laboratory Control	100	40.10 ± 1.20	100	52.0 ± 3.40
Latte Creek (Site Control)	100	41.20 ± 2.50	100	54.50 ± 2.30
Low	100	41.40 ± 1.80	100	50.60 ± 4.0
Moderate	95	39.20 ± 1.70	100	51.80 ± 3.20
High	100	39.50 ± 1.0	100	50.40 ± 1.90
Halfway Creek				
Laboratory Control	100	40.10 ± 1.20	100	40.10 ± 1.20
Halfway Creek (Site Control)	100	40.0 ± 3.10	100	40.0 ± 3.10
Low	100	39.0 ± 2.30	100	39.0 ± 2.30
Moderate	100	39.60 ± 1.80	100	39.60 ± 1.80
High	100	39.30 ± 0.30	100	39.30 ± 0.30

Notes: 1 -Expressed as mg of dry weight; Calculation of the survival standard deviation was not possible due to the lack of variability in the results

3.3.1; (Table 5). Although similar phosphorus and magnesium concentrations were measured in the creeks between seasons (Tables S2 and S3 Supplemental Material), the recorded stimulatory effect was slightly more noticeable in winter collected water for both, Latte and Halfway Creeks (Table 5). A significant reduction in growth ($p < 0.05$; ~ 15 % reduction compared to site control) was observed for algae exposed to low test concentrations using winter water collected from Latte Creek. However, these differences were not apparent at higher test concentrations (i.e., moderate and high; Table 5).

No effects on *C. dubia* survival were observed at any of the evaluated test concentrations (Table 6). A reduction in the number of produced broods (~25-30%) was observed for *C. dubia* exposed to moderate and high treatment concentrations for Latte Creek and Halfway Creek, respectively. Significant effects in winter water were not observed in the high exposure treatment in Latte Creek water, however a reduction in reproduction relative to site control is apparent. No effects occurred in rainbow trout survival and reproduction at any of the evaluated treatments and site waters (Table 7).

c) Metal mixture + Nutrients + TDS: General water quality parameters in Latte and Halfway Creeks are summarized in the Supplemental Material (Tables S6 and S7) and briefly described below. Water quality in exposure treatments remained constant through the tests and was within acceptability requirements for all evaluated species (as those shown in Table S1). Exposure water was generally characterized by circum neutral pH with maxima of 8.1. Dissolved oxygen levels were approximately 9.0 mg/L throughout the test (Table S6, Supplemental Material). Concentrations of ammonia and sulphate are described in the previous Section (3.3.2). The ammonia concentration was similar between creeks and seasons, however sulphate values appeared to be higher during the winter period for both creeks (Tables S2 and S3, Supplemental Material). Hardness values ranged approximately from 500 to 900 mg $CaCO_3$/L for Latte Creek and Halfway Creek waters collected in summer (Table S7 Supplemental Material). Conductivity values in the creek's exposure waters ranged approximately from 1,000 to 2,000µS/cm (Table S6, Supplemental Material). Similar to the b - Metal Mixtures and Nutrients study, conductivity values in exposure solutions increased relatively to site control due to salts and other inorganic chemicals added to achieve targeted treatment concentrations.

Results of the toxicity test using mixture solutions in Latte and Halfway Creek waters collected during the open water period are summarized in (Table 8). A stimulatory effect of site waters was noticeable for algal growth, which was consistent with the response recorded in previous tests (Section 3.3.1; Section 3.3.2). For *C. dubia*, no survival effects occurred in any of the evaluated treatment solutions however, reproductive impairment was evident in the moderate and high exposure in both Latte Creek and Halfway Creek waters. For rainbow trout, survival was not affected however, growth effects were evident in all low, moderate and high treatments in Latte Creek and Halfway Creek waters.

Discussion

The differences in background U concentrations between winter and summer collected site waters are related to naturally U-enriched groundwater discharges into the Latte and Halfway Creek systems. As previously reported (Muscatello, et al. 2020) [14] naturally elevated U concentrations are known to occur in groundwater within the Latte and Halfway Creek drainages. In winter, the base flows of the creeks are dominated by groundwater inputs, with negligible surface runoff input. Conversely, during the ice-free periods of the year (e.g., May to September), snowmelt and surface runoff inputs dilute the groundwater signature, resulting in considerably lower U concentrations in these creeks. These seasonal changes also have effects on other

Table 8: Test results (mean ± standard deviation) for *Pseudokirchneriella subcapitata, Ceriodaphnia dubia* and rainbow trout exposed to metal mixtures (arsenic, copper, chromium, uranium and zinc) + nutrients (sulphate and ammonia)+ total dissolved solids (TDS) solutions generated using Latte Creek and Halfway Creek collected in summer.

Treatments	*Pseudokirchneriella subcapitata*	*Ceriodaphnia dubia*		Rainbow trout	
	Cell Yield (x10^4 cells/ml)	Survival (%)	Reproduction (Number of broods)	Survival (%)	Growth[1] (mg)
Latte Creek					
Lab. Control	27.40 ± 1.70	100	17.50 ± 1.30	100	52 ± 3.40
Site Control	79.90 ± 3.30*	100	23.10 ± 3.80	100	54.50 ± 2.30
Low	77.80 ± 5.10*	100	21.90 ± 2.40	100	48.50 ± 1.50*#
Moderate	75.80 ± 4.80*	100	14.80 ± 4.50*#	100	49.80 ± 2.0*#
High	72.0 ± 4.80*	100	13.0 ± 5.10*#	100	50.70 ± 1.30*#
Halfway Creek					
Lab. Control	27.40 ± 1.70	100	18.10 ± 3.20	100	52.0 ± 3.40
Site Control	79.90 ± 3.30*	100	17.60 ± 7.80	100	54.50 ± 2.30
Low	77.80 ± 5.10*	100	17.70 ± 2.70	100	48.50 ± 1.50*#
Moderate	75.80 ± 4.80*	100	11.40 ± 2.50*#	100	49.80 ± 2.0*#
High	72.0 ± 4.80*	100	12.10 ± 2.20*#	100	50.70 ± 1.30*#

Notes: 1 - Expressed as mg of dry weight; * Significantly different ($p < 0.05$) from laboratory control; # Significantly different ($p < 0.05$) from site control.

parameters that have relevance to U bioavailability such as DOC. Most notably, concentrations of DOC are highest during the ice-free months (e.g., May to August in particular) due to terrestrial runoff.

In this study, a stimulatory growth effect was observed in all tests (i.e., tests a, b, c) for the algae *P. subcapitata*. This effect is mostly related to the natural presence of nutrients and more suitable growth media present in site water compared to laboratory water. The addition of nutrients such as ammonia and sulphate to the test water did not produce an increase in algal growth, with similar growth values reported for the a - Metal Mixture, b -Metal Mixture + Nutrients and c - Metal Mixture + Nutrients + TDS experiments. Winter water stimulated algae growth slightly more than collected summer water for both, Latte and Halfway Creeks. However, elements that may encourage algal growth such as for example magnesium and phosphorous, *P. subcapitata* exposed to spiked Latte and Halfway Creeks water is not immediately clear. A significant reduction in growth was showed similar values between seasons. Thus, the reason behind the observed stimulatory enhancement observed for algae exposed to the low concentration treatment using winter collected water from Latte Creek. However, these differences were not apparent at the high treatment concentrations and thus, assumed to be an artifact of the statistical analysis. The lack of adverse effects found in *P. subcapitata* exposed to metal mixtures (including U), sulphate, ammonia and TDS suggests that the algae may not be a suitable choice to evaluate (or derive) U thresholds applicable to aquatic environments downstream of planned (or operational) mines. Although no adverse effects were observed in *P. subcapitata* for all the evaluated tests (i.e., tests a, b, c) and exposure concentrations (i.e., low, moderate, high), the stimulatory growth enhancement observed to occur in site water may have implications for enrichment in receiving aquatic environments. This enrichment factor should be considered when evaluating potential effects in aquatic environments downstream of proposed and/or operational mines.

Previous studies reported by Muscatello, et al. (2020) [14] using Latte and Halfway Creek water spiked with U, postulated that the increased in DOC concentration present in the collected site water reduced the availability of U and hence, ameliorated its toxicity to *C. dubia*. For example, calculated toxicity endpoint concentrations for *C. dubia* (e.g., LC50 = 799 µg U/L; NOEC [reproduction] = 381 µg U /L) were several orders of magnitude higher than the applicable CCME WQGs for this element (short-term = 33 µg U/L; long-term = 15 µg U/L). Therefore, based on the highest exposure concentrations for U used in the present study (nominal concentration = 100 µg U/L) effects on the aquatic invertebrate were not truly expected to occur. It was postulated, however that additional metals in solution may alter the detoxification capacity in *C. dubia* at the expense of reproduction. When organisms face a trade-off between reproduction and survival imposed by a toxicant, in this case several metals in solution, they may reallocate resources toward detoxification mechanisms instead of reproduction and thus, achieve low mortality at the expense of reproductive fitness (Holloway, et al. 1990). It was expected that additional stressors in the a -Metal Mixtures test such as As, Cu, Cr and Zn may trigger detoxification mechanisms in *C. dubia.* These detoxification mechanisms are likely to be energy consuming and occur only at the expense of reproductive fitness (Holloway, et al. 1990; Muscatello and Liber 2009) [8] with reproductive effects occurring at lower U concentrations than those reported in previous studies (i.e., Muscatello et al. 2020) [14]. However, this did not appear to be the case in the present study as no adverse effects were recorded for *C. dubia* regardless of exposure to low, moderate and high metal mixtures. The concentrations of DOC in site waters are known to have a protective role against U toxicity responses in *C. dubia* (Muscatello, et al. 2020) [14]. Although not relevant for U (Goulet, et al. 2015) [6], it is known that metal toxicity is generally decreased by hardness (Borgmann, et al. 2005) [21]. It is plausible that DOC and hardness ranges in site waters play a protective role in ameliorating the toxicity response of *C. dubia* to metal mixtures of As, Cu, Cr, U and Zn. In contrast to single metals exposure, knowledge about metal mixture effects remains confounded. Interactions between the metals in treatment solutions, could also ameliorate toxicity. For example, effects on growth rate of *Lemna aequinoctialis* (duckweed) were lower when Cu and U were present in mixtures, relative to single/individual metal exposures, suggesting an antagonistic effect between these two elements (Charles, et al. 2006)[15] . It could be then concluded that mixtures of As, Cu, Cr and Zn with U at and/or below concentrations such as those reported in the high treatment exposure, are not expected to cause adverse effects to *C. dubia* in waters with similar chemistry characteristics (e.g., hardness, DOC) than those recorded for Latte and Halfway Creeks.

With the addition of sulphate and ammonia into the metal mixture (i.e., b - Metal Mixtures and Nutrients test) reproductive effects in *C. dubia* were apparent in winter water specifically for the moderate and high treatment in Latte Creek and Halfway Creek, respectively. Sulphate, and in minor degree nitrogen forms (i.e., ammonia), are contributors to the ionic composition of the water. Other major contributors to ionic strength are calcium and magnesium which play a main role in water hardness. The exposure water collected in winter from both creeks shows higher hardness (~ 600 mg $CaCO_3$/L) and conductivity (i.e., a measure of the ionic strength of water; ~1,000µS/cm) than those in summer (hardness ~400mg $CaCO_3$/L; conductivity ~700 µS/cm). It seems that *C. dubia* exhibited increased sensitivity to metal exposure in this winter water due to addition of sulphate and ammonia relative to the a -Metal Mixtures test. It is plausible the increased ionic strength of the test solutions generated using winter water (i.e., hard water and high conductivity) resulted in osmotic and ionic regulation challenges to the invertebrate that, combined with the evaluated metal concentrations, resulted in adverse reproductive effects at lower U concentrations (measured concentrations ~ 100 to 180 µg U/L for the moderate and high treatments, respectively) than those previously reported (NOEC ~ 300 µg U/L, Muscatello, et al. 2020 [14]. This has been reported to occur for other elements, for example, Elphick, et al. 2011, [22] reported increased sensitivity to sulphate in *C. dubia* at hardness values of 160 to 320 mgsulphate/L due to increased ionic strength of the water and osmotic challenges in the invertebrate. The addition of TDS (at nominal concentrations of 600, 800 and 1,000 mg TDS/L for the low, moderate and high treatments, respectively) to the metal and nutrient mixture (i.e., c -Metal Mixtures + Nutrients + TDS) produced the appearance of effects in *C. dubia* with reproductive impairment occurring in the moderate (~1,360and 1,220 mg TDS/L for Latte Creek and Halfway Creek, respectively) and high (~1,650and 1,580 mg TDS/L for Latte Creek and Halfway Creek, respectively) treatments for both creeks in summer collected water. These effect concentrations are in agreement with Weber-Scannell and Duffy (2007) [23] where TDS values ranging from 735 to 1,910 mg TDS/L produced adverse effects in exposed *C. dubia*. Two individual tests using summer collected Halfway Creek water spiked with TDS (nominal concentrations 200 to 1,500 mg TDS/L) and sulphate (nominal concentrations 50 to 1,000 mg/L) in combination with ammonia (at nominal concentrations of 2 mg/L [as N]) found no effects in exposed *C. dubia* with reported IC50 values above 1,500 mg/L and 1,000 mg/L, for TDS and sulphate, respectively (Muscatello and Flather 2021) [24]. This further substantiates the hypothesis that the effects observed in *C. dubia* exposed to a mixture of metals, ammonia, sulphate and TDS are the result of a decline in osmotic tolerances due to the increased ionic strength of exposure water, in association with an increased sensitivity to metal exposure triggered by the osmotic challenges faced by the invertebrate.

Exposure of swim-up rainbow trout fry to low, moderate and high metal mixtures, sulphate and ammonia solutions prepared using Latte Creek and Halfway Creek site water produced no observed reductions in survival or growth in winter or summer collected waters. Therefore, U in mixtures formulations of As, Cu, Cr, Zn, sulphate and ammonia at and/or below concentrations such as those reported in the high exposure treatment, are not expected to cause adverse effects to the fish specifically in waters with similar chemical characteristics (e.g., hardness, DOC) to those existing for Latte Creek and Halfway Creek. Rainbow trout is one of the most sensitive fish species to TDS exposure, particularly at the egg hardening stage and embryo developmental period (Weber Scannell and Jacobs 2001) [25]. Not surprisingly, the addition of TDS to Latte Creek and Halfway Creek waters (i.e., c -Metal Mixtures + Nutrients + TDS tests) triggered the appearance of significant growth reductions for all treatments (i.e., low, moderate and high) at measured TDS concentrations ranging from ~ 800 to 1,700 mg TDS/L. These effects are believed to be related to TDS rather than a result of exposure to metals (including U), sulphate and ammonia mixtures given the lack of effects recorded in these exposures (i.e., tests a and b). Tests using TDS in exposed salmonids have yielded mixed results, depending on site-specific conditions, ionic composition of exposure water, life stage and species used in the tests. For example, Chapman, et al. (2000),[26] reported no effects in rainbow trout (eggs and swim-up fry) at TDS concentrations > than 2,000 mg TDS/L whereas, Ketola, et al. (1988) [27] reported effects in survival of rainbow trout post fertilized eggs exposed to 1,500 mg TDS/L. Other authors reported reduction in the that fertilization and/or embryonic development of rainbow trout with IC25 values of 1,200 mg TDS/L and 600 mg TDS/L after 7 and 15 days of exposure, respectively. (Weber Scannell and Jacobs 2001) [23]. Based on the available scientific literature for rainbow trout exposed to TDS, the results reported in the present study fall within the concentration ranges expected (600 to 2,000 mg TDS/L) to cause adverse effects in this fish (independent of life stages evaluated) (Weber Scannell and Jacobs 2001) [23]. It should be noted that WQGs are not currently available for TDS. Guideline derivation should thus, incorporate evaluation of TDS effects to sensitive life stages (e.g., egg hardening) and sensitive species such as, rainbow trout. In addition, consideration of the ionic composition and strength of site waters should be considered as this may have an effect in toxicity responses.

Conclusion

Few studies have investigated interactions of U with other metals and elements, known to occur downstream of operational mines such as sulphate ammonia and TDS. The goal of the present study was to determine the effects of metal mixtures, ammonia, sulphate and TDS on the toxicity of U in *P. subcapitata, C. dubia* and rainbow trout. Three independent tests including a -b - Metal Mixtures, Metal Mixtures + Nutrients and c Metal Mixtures + Nutrients + TDS using spiked water collected from Latte Creek and Halfway Creek located in the YT, in summer and winter were performed to evaluate the presence of adverse effects. Organisms were exposed to three treatment solutions low, moderate and high per test. *P. subcapitata* showed no adverse effects for any of the evaluated tests (i.e.., tests a, b, c) and treatments (i.e., low, moderate and high). However, growth stimulation occurred in site water which may have implications for the enrichment of aquatic systems downstream planned and/operating mines. For *C. dubia*, no adverse effects were noted for the a -Metal mixtures test suggesting that U concentrations in combination with metals such as, As, Cu, Cr, Zn at and/or below concentrations such as those reported in the high exposure treatment, are not expected to cause adverse effects in waters with similar chemistry characteristics (e.g., hardness, DOC) than those existing for Latte Creek and Halfway Creek. The addition of ammonia and sulphate to the test solutions (i.e., b - Metal Mixtures + Nutrients) produced reproductive effects in winter collected water in both, Latte and Halfway Creek test solutions due to osmotic pressures faced by the invertebrate. Responses occurred at U concentrations lower (~100 to 180 µg U/L) than those previously reported to cause adverse effects in the invertebrate (NOEC ~ 300 µg U/L and postulated to be a result of increased sensitivity to metal exposures triggered by declines in the invertebrate osmotic tolerances. The addition of TDS to the mixture (i.e., c- Metal Mixtures + Nutrients + TDS) increased the appearance of reproductive effects in *C. dubia* and further supports the hypothesis that adverse effects are the result of a decline in the invertebrate's osmotic tolerances. For rainbow trout, adverse effects were only noticed for the c- Metal Mixtures + Nutrients + TDS test, and mostly a result of TDS in the moderate (measured concentration ~ 800 mg TDS/L) and high (measured concentration ~ 1,700mg TDS/L) treatments rather than metal exposure. Consideration of both the fresh water organism's sensitive life stages and the ionic strength of the water should be considered when deriving TDS WQGs.

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