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The Establishment of a New Culture of *Hyalella azteca* That Would Permit Toxicity Tests to Be Conducted on Low–Ionic Strength Waters

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Abstract: The objective of the present study was to establish a culture of *Hyalella azteca* that could be used for laboratory toxicity testing in low–ionic strength waters with electrical conductivities of <200 μS/cm. A wild strain of *H. azteca* was collected from Twin Lake, a small seepage lake with an electrical conductivity of 81 ± 27 μS/cm located on the property of Chalk River Laboratories in Chalk River, Canada. To determine the minimum aqueous ion requirements for an optimal culturing medium for the Twin Lake strain, Twin Lake was monitored for water quality and ionic content over 4 yr. Water quality parameters were averaged and used to formulate a medium containing $NaHCO_3$, $CaCl_2$, $MgSO_4$, KCl, NaBr, NaF, and LiCl, with an electrical conductivity of 89 ± 3 μS/cm. By evaluating survival and reproduction, it was concluded that this artificial medium promoted survival and supported reproduction (10 ± 4 neonates/female/wk) of the Twin Lake amphipod. The Twin Lake strain of *H. azteca* can, therefore, be maintained in laboratory settings, and this allows for toxicity testing to be conducted on low–ionic strength waters. *Environ Toxicol Chem* 2019;38:585–590. © 2019 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Hyalella azteca, Toxicity testing, Low–ionic strength water

INTRODUCTION

The Canadian Nuclear Laboratories' Chalk River Laboratories site is located in Chalk River, Ontario, Canada, within the Precambrian, Grenville Province of the Canadian Shield along the Ottawa River (Osei et al. 1997; Moltyaner and Killey 1988; Neymark et al. 2013). This area of the Ottawa River watershed is situated predominately on low solubility, metamorphic rock and thus, surrounding water waterbodies have a low alkalinity and low ionic strength.

Hyalella azteca is a common and important toxicity test species that is sensitive to metals (Bousfield 1958; De March 1977; Kruschwitz 1978; Nebeker et al. 1986; Mackie 1989; Borgmann et al. 2005, 1991, 1993; Phipps et al. 1995; Borgmann 1996; Maclean et al. 1996; Brooks et al. 2007). *Hyalella azteca* has been used in toxicity assessments by the Chalk River Laboratory Toxicity Laboratory for approximately 10 yr.

The Chalk River Laboratory Toxicity Laboratory has always obtained the epibenthic detritovore *H. azteca* from the Canada Centre for Inland Waters (Burlington, Ontario). It is known to be a cryptic species complex because, although morphologically similar, there are many genetically distinct clades of *H. azteca* which can vary in size and in sensitivity to toxicants (Leung et al. 2016). Twin Lake and Canada Centre for Inland Waters organisms are clade 1 (Leung et al. 2016; Walsh et al. 2017). The Chalk River Laboratory Toxicity Laboratory cultures and tests with these organisms were in a standard artificial medium (SAM), consisting of 5 salts (SAM-5S; $CaCl_2$, $NaHCO_3$, NaBr, KCl, and $MgSO_4$), which is representative of the water qualities found near Lake Ontario, where the culture originated (Borgmann 1996). The results of local sediment toxicity tests performed using the SAM-5S may not, however, be representative of toxicity effects in local water bodies on the Chalk River Laboratory site because of differences in water quality.

Previous studies have emphasized the importance of understanding the ionic content of test waters when studying the toxicity of contaminants in aquatic ecosystems because hardness mediates the toxicity of many metals (Maclean et al. 1996; Borgmann et al. 2005). Metal contaminants are generally more bioavailable to organisms in low–ionic strength waters because of the lack of natural ions competing for biological binding sites (Brooks et al. 2007). The watershed characteristics

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of the Chalk River Laboratory site are similar to many watersheds across Canada (Moltyaner and Killey 1988; Osei et al. 1997; Neymark et al. 2013). Soft water systems hold great ecological importance and are the habitat for many organisms, including a number of species at risk (Government of Canada 2017). For these reasons, the Chalk River Laboratory Toxicity Laboratory determined the need to establish a culture of *H. azteca* that could be maintained in low–ionic strength water to better represent toxicity in soft water systems (electrical conductivity $<200\ \mu S/cm$).

Over the years, attempts have been made to culture the Canada Centre for Inland Waters strain of *H. azteca* in various diluted standard artificial mediums and water from local water bodies (Ottawa River and Twin Lake waters); however, the Canada Centre for Inland Waters *H. azteca* did not reproduce in the softer water conditions, and testing was not possible (Walsh et al. 2017).

In 2010, a population of wild clade 1 *H. azteca* was collected from in Twin Lake, a water body located on the Chalk River Laboratory site (Figure 1). Similar to other lakes in the Ottawa River watershed, Twin Lake has low ionic properties. Since its discovery, the Chalk River Laboratory Toxicity Laboratory has performed a number of studies on Twin Lake *H. azteca*, including one examining copper toxicity in both Canada Centre for Inland Waters and Twin Lake strains of *H. azteca* (Richards et al. 2015). However, the wild *H. azteca* cultures were maintained in Twin Lake water, which proved to be problematic for a number of reasons. Firstly, the water had to be collected from the field, making laboratory testing impractical; secondly, seasonal variability in the water quality made testing inconsistent; and thirdly, there was the undesirable potential to bring parasites into the laboratory. Culturing the Twin Lake strain in the standard artificial medium water formulated for the Canada Centre for Inland Waters strain would not be ideal because it was not representative of conditions natural to Twin Lake *H. azteca*. Attempts at culturing Twin Lake *H. azteca* in diluted standard artificial medium yielded low reproduction compared to reproduction observed in the SAM-5S Canada Centre for Inland Waters culture (Walsh et al. 2017).

In 2015, a project was initiated to closely monitor the seasonal water qualities of Twin Lake to formulate a standard artificial medium that would closely match the natural water of Twin Lake to establish and maintain a healthy culture of *H. azteca* that could be used for toxicity testing in low–ionic strength waters.

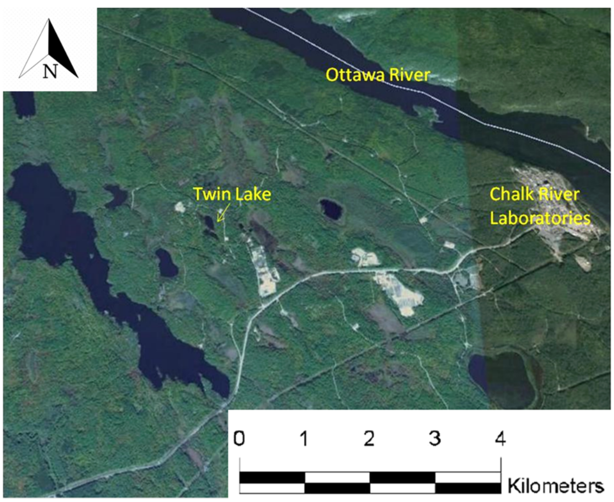

FIGURE 1: Location of Twin Lake (46°2′57.13″N, 77°24′50.91″W) on the Chalk River Laboratories site located in Ontario, Canada.

MATERIALS AND METHODS

Our laboratory performed 19 reference toxicity tests using copper sulfate over the duration of the present study. These 96-h reference toxicity tests were conducted using the Canada Centre for Inland Waters strain in SAM-5S water. The average lethal concentration to 50% of the organisms was 228 ± 53 mg/L (the lower warning limit was 138 mg/L, and the higher warning limit was 376 mg/L, 95% confidence interval) during the duration of the present study. No reference testing was completed using the Twin Lake *H. azteca* over the course of the present study; however, a comparison on the sensitivity of both strains to copper sulfate has been reported (Richards et al 2015).

To determine the acceptable range of pH, electrical conductivity, total hardness, dissolved oxygen, and inorganic ions for the Twin Lake *H. azteca* strain, a monthly water sample was collected from Twin Lake between July 2014 and July 2017 (unless ice conditions were unsafe or the lake was inaccessible). The pH and conductivity were measured using a Thermo Scientific Orion 3-star Bench Top meter and an Oakton Con 6 Acorn Series meter, respectively. Total hardness was measured using a direct reading titrator (Lamotte; 0–200 ppm). Dissolved oxygen was measured using a colorimetric Chemkit (Chemetrics). Ion analyses were completed using a Dionex-ion AS-DV chromatography system (Thermo Scientific).

When a sufficient amount of data was collected from Twin Lake (>1 yr), the results were used to produce trial standard artificial mediums. To ascertain the minimum aqueous requirements for the Twin Lake *H. azteca*, numerous standard artificial mediums were tested, and survival and reproduction were monitored in each medium.

The standard artificial medium used for Canada Centre for Inland Waters culturing, SAM-5S, was prepared according to Borgmann (1996) by adding 11.0 g $CaCl_2$, 8.4 g $NaHCO_3$, 3.0 g $MgSO_4$, 0.37 g KCl, and 0.12 g NaBr to 100 L of reverse osmosis water. Three diluted standard artificial mediums were formulated using a 1 to 7 ratio (electrical conductivity of SAM-5S is approximately 7 times greater than that of Twin Lake water) of standard artificial medium to reverse osmosis water in an attempt to simulate the salinity and ionic content of Twin Lake water. The first, 1:7 SAM-5S, was prepared by mixing one part SAM-5S with 7 parts reverse osmosis water. The second, 4-salt standard artificial medium (SAM-4S), was prepared by removing bromide to confirm the need for bromide. Diluted SAM-4S (1:7 SAM-4S) was prepared by mixing one part SAM-4S with 7 parts reverse osmosis water. The third, a diluted 7-salt (1:7 SAM-7S), was created, based on the water quality results obtained from the analysis of Twin Lake, by adding 3.85 g $CaCl_2$, 5.88 g $NaHCO_3$, 1.06 g $MgSO_4$, 0.25 g KCl, 0.1214 g NaBr, 0.0024 g NaF, and 0.0010 g LiCl to 35 L of reverse osmosis water and diluting the same as the other 2 standard artificial mediums. Table 1 provides a comparison of the pH, conductivity, total hardness, and ionic content measured in all water types.

TABLE 1: Comparison of the water quality and ion content data measured for Twin Lake water (average) and the 4 standard artificial media[a]

	Water type				
Parameter	SAM-5S	1:7 SAM-5S	1:7 SAM-4S	1:7 SAM-7S[b]	TL
pH	8.1	7.7	8	7.6	6.7 ± 0.4
Conductivity (μS/cm)	403	64	67	90	81 ± 27
Total hardness (as $CaCO_3$) (mg/L)	66	12	7	12	12 ± 4
Cl^- (mg/L)	96.56	10.22	11.04	10.66	10.48 ± 6.80
Br^- (mg/L)	0.82	0.15	<0.06	0.35	<0.06
NO_3^- (mg/L)	<0.07	<0.07	<0.07	0.03	0.14 ± 0.10
N (mg/L)	<0.01	<0.01	<0.01	0.01	0.03 ± 0.02
SO_4^- (mg/L)	25.01	3.50	3.31	3.41	2.08 ± 1.12
PO_4^- (mg/L)	<0.05	<0.05	<0.05	0.27	0.30 ± 0.37
F^- (mg/L)	<0.04	<0.04	<0.04	0.02	0.03 ± 0.03
Na^+ (mg/L)	23.52	4.25	7.67	9.08	5.91 ± 2.36
Ca^{2+} (mg/L)	38.68	6.33	3.70	7.05	3.66 ± 1.29
Mg^{2+} (mg/L)	5.97	0.78	0.75	0.89	1.40 ± 0.34
K^+ (mg/L)	1.86	0.80	0.53	0.50	0.92 ± 0.35
Li^+ (mg/L)	<0.0011	<0.0011	<0.0011	0.0017	0.0013 ± 0.001

[a]Standard artificial media (SAM: 4S = 4 salts; 5S = 5 salts; 7S = 7 salts), and Twin Lake water composition are listed. The standard artificial media parameter values are not averages because they are based on precalculated compositions, and inductively coupled plasma-mass spectrometry was used to verify the composition of the standard artificial medias.
[b]The detection limits for inductively coupled plasma-mass spectrometry were lower for the 1:7 SAM-7S analysis compared to the other standard artificial medias.
TL = Twin Lake.

Adult *H. azteca* were collected from Twin Lake in 2011 and identified as clade 1; the analysis was completed on 5 organisms. From September 2014 to September 2017, an additional 500 *H. azteca* were collected from Twin Lake. The amphipods were collected close to shore using dip nets, which were dragged through aquatic vegetation and along the lake bed. The organisms were transferred to the laboratory and allowed to acclimatize for 2 wk in filtered Twin Lake water (0.45 μm, high-capacity disposable filter; Geotech). The water was filtered through a high-capacity filter to remove predators and parasites, and a microscope was used to confirm their removal. Following the acclimatization period and confirmation that no parasites infected the wild amphipods, the *H. azteca* were acclimatized to a predetermined standard artificial medium treatment and transferred to the Chalk River Laboratory Toxicity Laboratory. Then, Canada Centre for Inland Waters *H. azteca* were placed in SAM-5S, 1:7 SAM-5S, 1:7 SAM-4S, and Twin Lake water; and the Twin Lake organisms were tested in Twin Lake water, 1:7 SAM-7S, 1:7 SAM-5S, and 1:7 SAM-4S. The acclimatization to each water type was done gradually over 48 h, with no associated mortality. Both organisms where first placed in 50% for 24 h and then 75% for 24 h of the culture water that they would be tested in.

All *H. azteca* (Twin Lake and Canada Centre for Inland Waters) treatments were cultured at 23 ± 3 °C, and the light intensity was approximately 850 lux at the surface of the culture aquaria (16:8-h light: dark cycle). Each culture aquarium was oxygenated and supplied with a piece of 300-μm Nitex® mesh for the organisms to cling to, and organisms were fed ground Tetramin® fish flake, 3 times weekly (~ 0.5 mg/individual). Cultures were examined daily for mortality and signs of reproduction (mating pairs), and the amphipods were kept in the trial standard artificial mediums for at least 1 mo prior to assessing reproduction. The organisms were removed from the trial standard artificial medium if high mortality rates were observed or there were no signs of reproduction for a period exceeding 1 mo.

Following this acclimatization period, if mating pairs were observed, they were transferred to separate oxygenated 1.9-L buckets of corresponding standard artificial medium treatment water (each containing a 9-cm^2 300-μm Nitex mesh) to closely monitor reproduction. The pairs did not detach during the transfers. The temperature, light intensity/cycle, and feeding regime were the same as for the culturing conditions. Fifty percent of the water was replaced weekly at the same time that juveniles were screened and removed. The water chemistry was monitored just before the water changes. Temperature, dissolve oxygen, ammonia, nitrite, pH, conductivity, and general hardness were measured.

To initiate the 7-d reproduction monitoring, a minimum of 5 mating pairs (10 adults) were required. If there were greater than 15 mating pairs, a new bucket was initiated for that trial standard artificial medium. Cultures of Canada Centre for Inland Waters *H. azteca* in SAM-5S and Twin Lake *H. azteca* in 1:7 SAM-7S are still being maintained at the Chalk River Laboratory Toxicity Laboratory.

The Mann-Whitney rank sum test and a normality test (Shapiro-Wilk) were used to evaluate differences in reproduction data between the various test waters. The conclusions of the statistical tests were based on 2-sided tests at the 0.05 significance level.

RESULTS AND DISCUSSION

Monitoring Twin Lake between 2013 and 2017 provided data on variations in water quality. The pH remained fairly constant over the years at 6.7 ± 0.4. Total hardness (as $CaCO_3$) ranged from 5 to 18 mg/L and followed the same trend as conductivity (36–106 μS/cm; Figure 2), and both decreased with water levels during the summer months. Figure 3 provides a visualization of the seasonal cycling of chloride and sodium concentrations. This pattern was the same for all detectable ions in Twin Lake water.

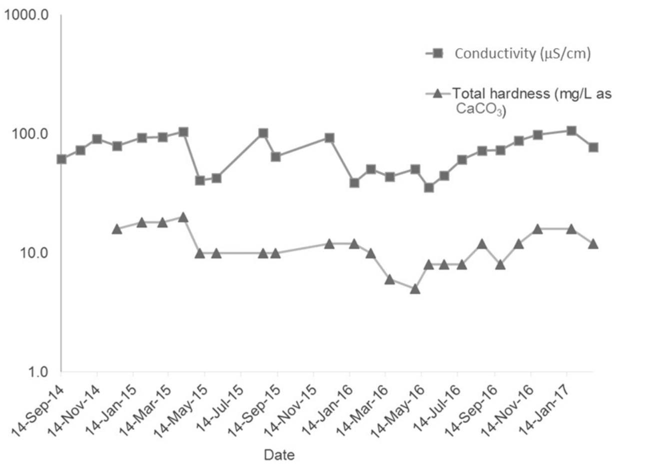

FIGURE 2: Comparison of Twin Lake water conductivity and total hardness measurements over a 4-yr time period. Also refer to Supplemental Data, Figure S1.

Other detectable ions in Twin Lake water and their concentration ranges are as follows: sulfate (0.15–3.81 mg/L), nitrate (0.03–0.32 mg/L), nitrogen (0.01–0.07 mg/L), fluoride (0.01–0.09 mg/L), calcium (1.80–6.98 mg/L), magnesium (2.11–0.79 mg/L), and potassium (0.54–1.98 mg/L).

Data collected from the monitoring of Twin Lake water show that although pH remains consistent, ionic content, conductivity, and hardness undergo seasonal changes that repeat yearly. The mentioned parameters tend to increase in the fall and winter months and to decrease in the spring and summer months. The decreases measured in the spring and summer may be attributed to snow melt and rainfall increasing the water level of the lake, thereby diluting the ions in the water. The high levels of calcium and sodium in Twin Lake are likely the result of road salt application because the main access road to Chalk River Laboratory is located 900 m away from Twin Lake.

The identification of the Twin Lake *H. azteca* as clade 1, the same clade as the Canada Centre for Inland Waters culture, has interesting implications for potential toxicity test comparisons in the future. Studies have shown that different clades of *H. azteca* differ in their sensitivity to contaminants (Leung et al. 2016). The Canada Centre for Inland Waters *H. azteca* were followed for at least 1 yr in SAM-5S (6 replicates), Twin Lake water (2 replicates), and 1:7 SAM-4S (2 replicates) with a mortality rate of approximately 0.1% per day (Table 2). Considering *H. azteca*'s reported longevity in laboratory cultures, these rates are considered normal (Hargrave 2011). The Canada Centre for Inland Waters organisms placed in 1:7 SAM-5S (2 replicates) were followed for 6 mo and presented a mortality rate of approximately 0.2% per day. The highest average reproduction rate was obtained in SAM-5S. The average rate was cut in half when the amphipods were placed in 1:7 SAM-5S, reduced by a factor approaching 10 when in 1:7 SAM-4S, and reduced by 30-fold in Twin Lake water.

As shown in Table 2, Twin Lake *H. azteca* showed a mortality rate comparable to the standard Canada Centre for Inland Waters culture when kept in Twin Lake water and in 1:7 SAM-7S (observations on duplicate buckets recorded over at least 1 yr). The Twin Lake strain was found to die off when cultured in 1:7 SAM-5S and 1:7 SAM-4S (Table 2). The same was noted when the lithium was removed from the SAM-7S (Table 2). Reproduction data for the Twin Lake strain are displayed in Figure 4 as the average number of neonates (juvenile *H. azteca*) per female per week for Canada Centre for Inland Waters *H. azteca* in SAM-5S and Twin Lake *H. azteca* in all water types. Diluted SAM-5S was prepared to match the conductivity and hardness of Twin Lake water. This was the first step in formulating a low–ionic strength standard artificial medium; however, as seen in Figure 4, this standard artificial medium was not ideal for Twin Lake *H. azteca*, and the reproductive success was 3 ± 2 neonates/female/wk, 3 times less than the Canada Centre for Inland Waters strain in SAM-5S. The difference between the 2 groups was statistically significant ($p = 0.047$). Next, we assessed if bromide was necessary for Twin Lake *H. azteca*, as it is for Canada Centre for Inland Waters *H. azteca* (Borgmann 1996). As seen in Figure 4, the Twin Lake organisms had very low reproduction rates in 1:7 SAM-4S (<1 neonate/female/wk), suggesting that the Twin Lake strain also benefits from the addition of bromide. Finally, 1:7 SAM-7S was formulated such that water chemistry and quality were closely related to Twin Lake water but included bromide to enhance the culture's health and reproduction. Figure 4 shows the reproduction rate of 10 ± 4 neonates/female/wk compared to other media and to the Canada Centre for Inland Waters strain in SAM-5S (6 ± 2 neonates/female/wk). The Twin Lake amphipods in 1:7 SAM-7S had, on average, significantly higher reproductive success when compared to Canada Centre for Inland Waters *H. azteca* in SAM-5S and Twin Lake *H. azteca* in Twin Lake water ($p = 0.016$ and < 0.001, respectively). Figure 4 depicts the differences in reproductive

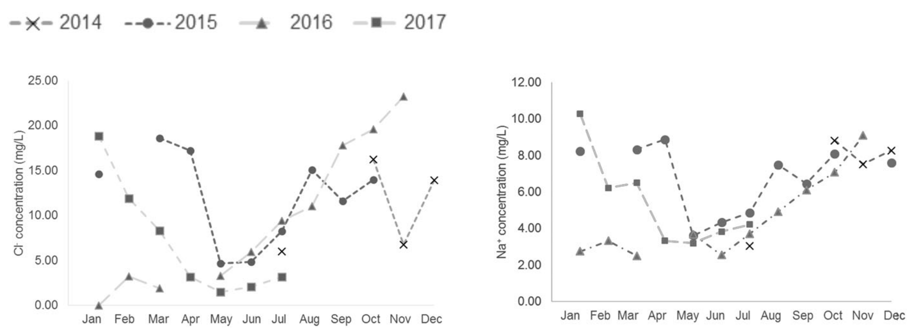

FIGURE 3: Chloride and sodium concentrations in Twin Lake water over 4 yr.

TABLE 2: Percentage of Canada Centre for Inland Waters and Twin Lake organisms lost daily in each water type tested

Water type	CCIW	TL
SAM-5S	0.09	
TL	0.10	0.07
1:7 SAM-4S	0.10	1.09
1:7 SAM-5S	0.25	2.71
1:7 SAM-7S, no lithium		1.10
1:7 SAM-7S		0.16

CIW = Canada Centre for Inland Waters; SAM = standard artificial media (4S = 4 salts; 5S = 5 salts; 7S = 7 salts); TL = Twin Lake.

success observed for Twin Lake *H. azteca* in 1:7 SAM-7S and the 2 other diluted standard artificial mediums (1:7 SAM-5S and 1:7 SAM-4S).

As previously mentioned, Twin Lake does not have detectable bromide concentrations (<0.06 mg/L). This indicates that there is a component of Twin Lake water that is missing from the present analyses that has the same beneficial properties as bromide for *H. azteca*. Previous studies examining the influence of iodide on *H. azteca* have reported similar culture successes with the addition of iodide in the absence of bromide (Borgmann 2002; Ivey et al. 2011). The Canadian Nuclear Laboratory has tested for iodide in Twin Lake water; however, concentrations were below the detection levels (0.02 μg/mL) using the current methods. A study by Gilfedder et al. (2008) suggests that iodide is a preferred species for biological uptake and that, therefore, aqueous concentrations are quite low in freshwater systems (0.0001 μg/mL), with higher concentrations generally measured in plant and animal (algae and zooplankton) organisms. It is possible that Twin Lake *H. azteca* are consuming iodide present in algae and plant matter and thereby thrive in the wild without the presence of bromide or iodide in the water. Moving forward, the specific chemical and microbiological transformations of iodine species in Twin Lake water will be studied. In the future, it may be possible to remove bromide from the SAM-7S if the current protein-based diet is changed to a diet rich in iodide. Additional work will also include verifying the importance of lithium to the Twin Lake *H. azteca* to determine if it is a necessary component of the standard artificial medium. At the moment, without lithium addition, the cultures are not thriving (Table 2).

In the present study, we have shown that the 1:7 SAM-7S formula promotes the health and reproduction of Twin Lake *H. azteca*. It is therefore possible to maintain a strong culture of Twin Lake *H. azteca* in the laboratory setting and thereby have consistent and reliable access to *H. azteca* appropriate for the testing of toxicity in low–ionic strength waters. The results of such tests have the potential to give Canadians and those in other countries with low–ionic strength waters (<200 μS/cm) the ability to obtain relevant toxicity, bioavailability, and bioaccumulation data to evaluate risk associated with contaminant exposure.

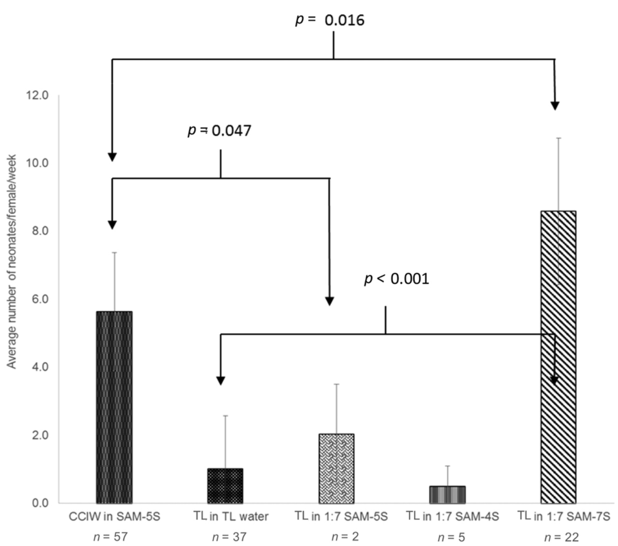

FIGURE 4: Comparison of reproduction of Canada Centre for Inland Waters *Hyalella azteca* in standard artificial medium (SAM)-5 salts and Twin Lake *H. azteca* in Twin Lake water and 3 diluted standard artificial mediums. The numbers of replicate measurements for each treatment group were 57, 37, 2, 5, and 22, respectively. Error bars indicate 1 σ. CCIW = Canada Centre for Inland Waters; 4S = 4 salts; 5S = 5 salts; 7S = 7 salts; TL = Twin Lake.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4348.

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Data Accessibility—Data, associated metadata, and calculation tools are available from the corresponding author (marilyne.stuart@cnl.ca).

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