Phylogenetic analysis of *Hyalella* colonization in lakes recovering from acidification and metal contamination

J.J. Babin-Fenske, T.J.S. Merritt, J.M. Gunn, T. Walsh, and D. Lesbarrères

Abstract: The freshwater amphipod *Hyalella azteca* (Saussure, 1858) is common throughout North America and is a popular organism for toxicity tests and assessment of an ecosystem's health. However, recent studies suggest that this "species" may actually be a number of closely related species, possibly with distinct habitat requirements. The region in and around Sudbury, Ontario, has many lakes recovering from acidification and metal contamination with *Hyalella* slowly recolonizing the area. Analyzing mitochondrial cytochrome *c* oxidase I (*COI*) sequences, we find two major groups of *Hyalella*: one group associated with recolonization of the central, historically more contaminated set of lakes and a second group associated with the more distant, less impacted, lakes. Morphologically, these inner city amphipods are significantly larger than those observed in lakes farther away from the city. This study may provide a foundation for a better understanding of *Hyalella* species complex, as well as colonization routes, toxicological sensitivities, habitat requirements, and dispersal capabilities.

Key words: amphipod, *Hyalella azteca*, colonizing species, acidification, lake recovery, industrial pollution.

Résumé : L'amphipode d'eau douce *Hyalella azteca* (Saussure, 1858) est répandu dans l'ensemble de l'Amérique du Nord et c'est un organisme couramment utilisé pour les études de toxicité et l'évaluation de la santé des écosystèmes. Cependant, les études récentes suggèrent que cette « espèce » pourrait en fait être un ensemble d'espèces étroitement liées, probablement avec des besoins écologiques distincts. La région de Sudbury (Ontario) et ses alentours comptent beaucoup de lacs en voie de rétablissement à la suite de l'acidification et de la contamination par des métaux, et *Hyalella* recolonise lentement le secteur. En analysant des séquences du génome mitochondrial, la cytochrome *c* oxydase (*COI*), nous avons trouvé deux groupes importants de *Hyalella* : un groupe associé à la recolonisation de la région centrale, qui comprend historiquement un ensemble de lacs plus contaminés, et un deuxième groupe associé aux lacs plus éloignés et moins touchés par la contamination historique. Morphologiquement, les amphipodes du centre urbain sont significativement plus grands que ceux observés dans les lacs plus éloignés de la ville. Cette étude peut fournir les bases d'une meilleure compréhension de la lignée *Hyalella* ainsi que des voies de colonisation, des sensibilités toxicologiques, des besoins en matière d'habitat et des capacités de dispersion de ces organismes.

Mots-clés : amphipode, *Hyalella azteca*, espèces colonisatrices, acidification, amélioration de la qualité des lacs, pollution industrielle.

Introduction

The freshwater amphipod *Hyalella azteca* (Saussure, 1858) is common throughout North America and is a popular organism for toxicity tests and assessment of an ecosystem's health (Environment Canada 1997; USEPA 2000; Tao et al. 2010). However, interpretation of toxicological results has become more complicated after genetic analyses indicated multiple evolutionary lineages within *Hyalella azteca*, suggesting that this group is a species complex, not a discrete species (Duan et al. 1997; Hogg et al. 1998; Witt and Hebert 2000). Recently, Witt et al. (2006) suggested there may be over 30 species within the complex of *H. azteca* based on an analysis of variation in the mitochondrial cytochrome *c* oxidase I (*COI*) gene across 49 sites in the southern Great Basin region of California and Nevada. Morphological and ecological data have also suggested distinct populations in Oregon, Oklahoma, and Michigan (Strong 1972; Wellborn et al. 2005). We propose that the combination of molecular and morphological analyses may facilitate further investigation of the *Hyalella* species complex and provide insight into distribution and habitat requirements, e.g., in long-term monitoring of the reestablishment of *Hyalella* into lakes recovering from pollution.

The current distribution of *Hyalella* appears to reflect genetic, morphological, and ecological differentiation of specific lineages. Determining the factors that limit, or promote, colonization of recovering environments is a fundamental

Received 27 September 2011. Accepted 13 February 2012. Published at www.nrcresearchpress.com/cjz on 21 April 2012.

J.J. Babin-Fenske,* J.M. Gunn,* T. Walsh,* and D. Lesbarrères.* Department of Biology, Laurentian University, Sudbury, ON P3E 3C6, Canada.
T.J.S. Merritt.* Department of Chemistry and Biochemistry, Laurentian University, Sudbury, ON P3E 2C6, Canada.

Corresponding author: Thomas Merritt (e-mail: tmerritt@laurentian.ca).

*The authors contributed equally to this work.

doi:10.1139/Z2012-028

challenge in modern zoology. Establishment of *Hyalella* into polluted lakes may be limited by both toxicological factors and ecological variables. Wellborn et al. (2005) found a correlation between genotypes, size, and predation in which lakes without fish predators had larger bodied genotypes, while lakes with predatory fish had smaller bodied genotypes. The city of Greater Sudbury, Ontario, is an interesting study site to further examine this apparent connection between genotype and morphology in *Hyalella* colonization because the area contains lakes without *Hyalella*, lakes recently colonized by the amphipod, and lakes with well-established populations (J.J. Babin-Fenske, unpublished data). These lakes also have a reduced fish and benthic invertebrate diversity owing to the environmental impacts of decades of logging, mining, and smelting activities (Keller et al. 2007; Wesolek et al. 2010). The effects of the smelting complexes resulted in over 7 000 lakes within a 17 000 km^2 area to be acidified to the point of biological damage (Neary et al. 1990). Although the reduction of approximately 90% of sulfur and metal emissions began in the 1970s (Potvin and Negusanti 1995), the landscape surrounding the three main smelting complexes remains one of the largest areas of industrial barrens in the world, as described by Kozlov and Zvereva (2007). Lakes in the region are slowly recovering with increased pH, lower metal concentrations, and the reestablishment of sensitive aquatic species (Keller et al. 2007). Lake recovery in this area has been, and continues to be, well studied in terms of changes in water chemistry and species richness, including both vertebrate (e.g., fish) and invertebrate species. *Hyalella* colonization is of particular interest, because of the use of this group for toxicological studies and the suggestion from previous research on other locations that multiple genetic lineages are likely present. As lakes become more habitable for *Hyalella*, new populations may be founded by immigration of lineages from geographically distant areas or by more local populations expanding distribution.

The objective of this study was to examine the genetic and morphological characteristics of *Hyalella* populations reestablishing in the industrially damaged region of Sudbury and to determine the diversity of lineages recolonizing the region. Although little is known about the habitat requirements of the various *Hyalella* genotypes, we suspected that different lineages could be more or less successful at colonizing the recovering lakes of the Sudbury area. Specifically, we hypothesized that the different recent histories of the lakes in our study could be reflected in the presence or absence of large and small ecomorphs recently identified within *Hyalella azteca* (Wellborn et al. 2005; Wellborn and Broughton 2008). We sequenced a fragment of the *COI* gene from 104 individuals from 15 lakes in and around the Sudbury area. In a phylogenetic analysis of these sequences, we find that all specimens collected from study lakes within 30 km of the Sudbury city centre were of a single lineage including only large-bodied *Hyalella* described from previous studies in the United States (Wellborn et al. 2005; Wellborn and Broughton 2008). Morphological analyses suggested that this group of specimens were significantly larger than the specimens found outside of the city. To our knowledge, this study is the first to examine the colonization by *Hyalella* sp. in an industrially damaged habitat and provides a foundation for further studies on habitat requirements and descriptions of these sensitive indicator invertebrates.

Materials and methods

Specimen collection and preparation

Specimens of *Hyalella* were collected in 2005, 2007, and 2010 using a kick-sweep method from 14 lakes within Ontario, Canada. Nine of these lakes were located within 30 km of the city centre of Greater Sudbury, four lakes were located 30–90 km north of the city, and one lake was 52 km south (inner and outer lakes respectively; Table 1). All collections occurred in late summer and early fall. Samples were also taken from the Canadian Centre of Inland Waters (CCIW) laboratory population used in toxicological studies. The original CCIW colony was established from a collection from Valens Conservation Area, Ontario, over 350 km south of Sudbury. Specimens were submitted to the Canadian Centre for DNA Barcoding (CCDB) at the Biodiversity Institute of Ontario at the University of Guelph for molecular analysis. Three of the city lakes have recently been colonized with the first recent appearance of *Hyalella* in 2003 (Hannah Lake) and 2010 (Daisy and Lohi lakes). Select specimens collected in 2010 were photographed and one half of the body was retained in 95% ethanol in a –20 °C freezer for additional analyses and measurements. COI barcodes were acquired from the CCDB using standard barcoding protocols for DNA extraction (Ivanova et al. 2006, 2007), polymerase chain reaction (PCR) amplification (Ivanova and Grainger 2007*a*), and sequencing (Ivanova and Grainger 2007*b*).

Sequences and photographs are available on the Barcode of Life Data systems Web site (Ratnasingham and Hebert 2007; http://www.barcodinglife.com/views/login.php, accessed 15 February 2012) within the projects TWAMI “Greater Sudbury Amphipod-*Hyallela* Analysis” and HOSO “*Hyalella* of Sudbury Ontario”. Additional DNA sequences were retrieved from GenBank including the outgroup (*Diporeia hoyi* (Bousfield, 1989) and *Platorchestia platensis* (Krøyer, 1845)) and *Hyalella* specimens examined by Wellborn and Broughton (2008), accession Nos. EU621724–EU621762.

Molecular analyses

DNA sequences were aligned with ClustalW, implemented in MEGA version 4 (Tamura et al. 2007) and short, or fragmented, sequences were removed for analyses. Bayesian analyses were implemented with MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) using gamma-distributed rate variation across sites and a proportion of invariable sites with HKY+I+G model, as selected by jModeltest version 0.1.1 (Posada 2008). Analyses were run for 3 000 000 generations. Maximum likelihood analyses were performed using the online version of RAxML (available from http://phylobench.vital-it.ch/raxml-bb/index.php, accessed 15 February 2012) (Stamatakis et al. 2008) with GTR+I+G parameters. Pairwise nucleotide sequence divergence for mean intra- and inter-clade comparisons was calculated using the Jukes–Cantor estimate in MEGA version 4.0 (Tamura et al. 2007).

Morphological analyses

Head length was measured for females collected in 2010

Table 1. Collection locations of *Hyalella* in Ontario for this study.

Lake	Distance from Sudbury city centre (km)	Latitude (N)	Longitude (E)	Clade
Inner lakes				
Hannah Lake*,†	6	46°26′45.60″	–81°2′6.01″	Inner
Middle Lake	6	46°26′16.80″	–81°1′30.00″	Inner
Inco Flux Pit	8	46°28′4.80″	–81°5′16.80″	Inner
Richard Lake†	9	46°25′55.20″	–80°56′2.39″	Inner
Daisy Lake*,†	10	46°26′38.40″	–80°54′3.60″	Inner
Raft Lake	10	46°24′54.00″	–80°55′51.60″	Inner
Lohi Lake*,†	12	46°22′58.80″	–81°1′58.79″	Inner
Long Lake	12	46°24′25.20″	–81°1′4.80″	Inner
Garson Flux 10-2 Pit	13	46°34′12″	–80°52′12.01″	Inner
Outer lakes				
Fairbank Lake	32	46°28′8.41″	–81°25′30.01″	Outer B, D, E
Windy Lake†	35	46°35′49.20″	–81°24′43.21″	Outer B, C, E
Geneva Lake†	51	46°45′28.80″	–81°33′21.60″	Outer B
Carlyle Lake†	52	46°3′10.80”	–81°18′10.80″	Outer B
Low Water Lake†	91	47°7′8.40″	–81°42′10.81″	Outer A, B, C
Laboratory				
Norwood Laboratory, Valens Conservation Area, Ontario†	351	43°22′46.28″	–80°8′26.80″	Inner

Note: Clade designation refers to groups described in the text and presented in Table 2 and Fig. 1.

*Denotes lakes that have been recently colonized by *Hyalella*.

†Denotes locations sampled or resampled in 2010.

Fig. 1. Phylogenetic tree of *Hyalella* of the Sudbury region (Outer A–E and Inner), a laboratory population (Lab), and ecomorph clades (Small A–C, Large OK, and Large MI/OR) from Wellborn and Broughton (2008). Bayesian posterior probabilities (first value) and bootstrap values for the maximum likelihood analysis (second value) are shown at each node. Numbers in parentheses and pie charts represent the number or percentage of sequences for each group. The scale bar represents the number of nucleotide substitutions per site.

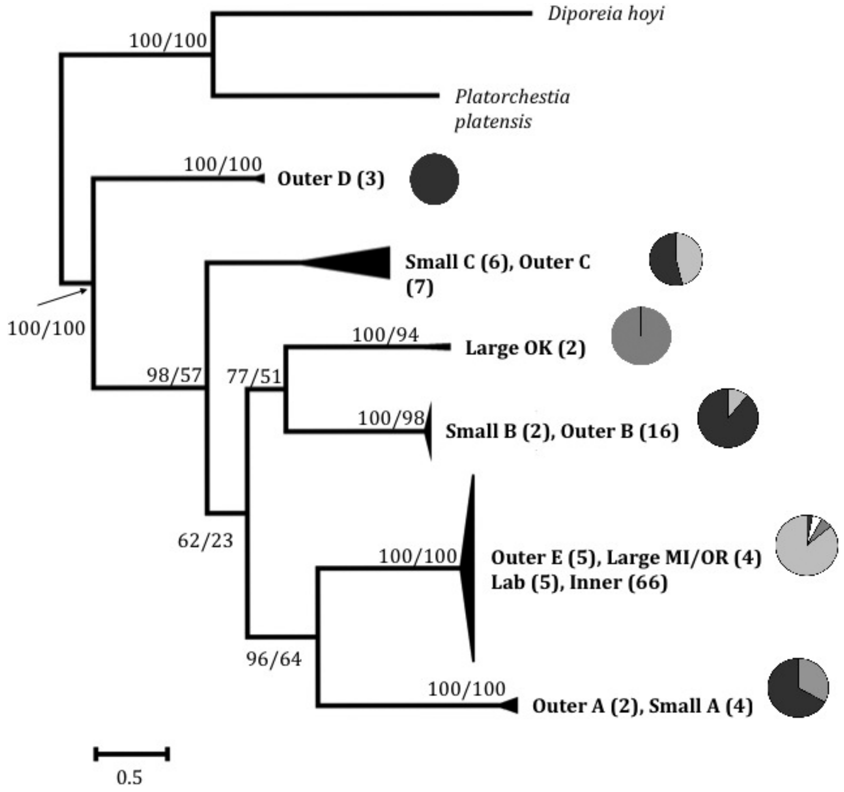


Table 2. Jukes–Cantor estimates for mean intra- and inter-clade sequence divergence with standard error (SE).

Interclade sequence divergence	Inner	Outer A	Outer B	Outer C	Outer D	Large OK	Intraclade sequence divergence	SE
Inner							0.012	0.002
Outer A	0.22						0.011	0.003
Outer B	0.221	0.24					0.006	0.002
Outer C	0.249	0.231	0.202				0.049	0.006
Outer D	0.262	0.257	0.267	0.258			0.005	0.003
Large OK	0.235	0.235	0.192	0.22	0.259		0.038	0.008
Outgroup	0.356	0.341	0.329	0.331	0.327	0.316	0.278	0.024

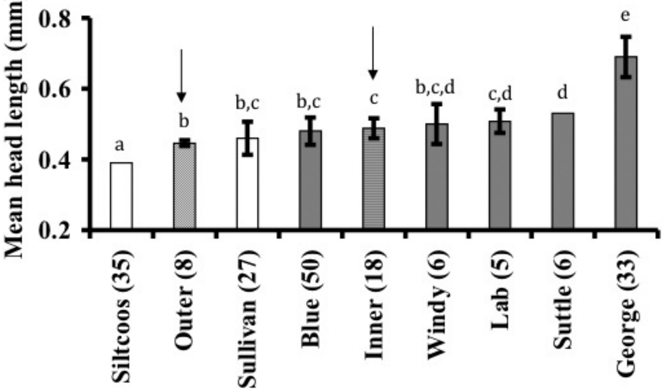
Note: Minimum and maximum values are indicated in grey.

and used as an index of body size following Strong (1972) and Wellborn et al. (2005). Only gravid females were examined to ensure that only mature specimens were included in our morphological analysis. This restriction limited the number of specimens available for analysis, so given our small sample size, lakes were not analyzed separately but were grouped by location into inner and outer region lakes for this part of the study. Outer lakes included in these analyses were Low Water Lake, Geneva Lake, and Carlyle Lake. A Student's *t* test was performed to compare the size of specimens collected within 30 km of the city centre (inner lake specimens) to those collected outside of city limits (outer lake specimens). Windy Lake and Fairbank Lake were considered borderline lakes (32 and 35 km from city centre, respectively) and appeared to have a mixture of both small and large ecomorphs. Specimens from Windy Lake were therefore separated for morphological statistical analyses, whereas no specimens from Fairbank Lake were available for morphological analyses. Reared specimens from the CCIW laboratory were also analysed separately from other inner specimens for analyses because such laboratory populations may have altered morphology or genetic structure (Mackauer 1976).

Results

Phylogenetic analyses identified six major clades that are well supported by both Bayesian posterior probabilities and maximum likelihood bootstrap support (Fig. 1). These groups correspond closely to the clades described in Wellborn and Broughton (2008), with an extra clade of three specimens from Fairbank Lake that has no other affiliation. Outer Sudbury specimens formed five clades and were associated with smaller ecomorphs from Michigan, Oregon, and Oklahoma. Inner Sudbury specimens formed a single clade and were associated with large ecomorphs described earlier from Michigan and Oregon. This inner clade also included the laboratory specimens and five outer specimens from Windy Lake and Fairbank Lake. Mean intraclade divergence ranged from 0.5% to 4.9%, whereas mean interclade divergence ranged from 19.2% to 26.7% (Table 2).

Fig. 2. Mean head length (mm) of *Hyalella* collected in this study (Outer and Inner) and measurements from past publications: Siltcoos and Suttle (Strong 1972); Sullivan, Blue, and George used as extremes for each ecomorph (Wellborn 1995; Wellborn et al. 2005). Error bars represent ±1 SD and numbers in parentheses are sample sizes. Dark grey bars represent the specimens deemed large ecomorph and white bars represent the small ecomorph. Sudbury inner and outer specimens are indicated by arrows. Windy Lake and Lab specimens are labeled but separated from other groups (for details refer to the Morphological analyses subsection of the Materials and methods). Lowercase letters indicate Student's *t* test significance at $p < 0.05$.



Head length was significantly shorter in outer Sudbury specimens than inner specimens (Student's *t* test, $t = 5.91$, $p < 0.001$). Windy Lake and CCIW laboratory specimens had a mean length of 0.500 mm and 0.508 mm, respectively, which is larger than the mean length of 0.488 mm for the inner larger specimens. Windy Lake specimens had a large standard deviation and were not significantly different from either the small or the large ecomorph. Comparison with measurements from past publications demonstrate that the mean head length of outer Sudbury specimens lies in the range of specimens deemed the small ecomorph through molecular analyses (Fig. 2). By contrast, the inner Sudbury specimens have a mean head length within the range of specimens designated as the large ecomorph.

Discussion

Molecular analyses provide evidence that a single phylogenetic clade of *Hyalella* is colonizing the once-polluted lakes of Sudbury. These amphipods are significantly larger than those found in lakes farther away from the city. Our phylogenetic analysis indicates that these large-bodied amphipods are closely related to the Michigan–Oregon large ecomorph described by Wellborn and Broughton (2008), and therefore do not represent an independent derivation of this large-bodied phenotype. All specimens found within 30 km of Sudbury centre were associated with the large genotype clade, whereas all specimens found farther than 50 km of Sudbury were in the smaller genotype clades. Specimens from borderline lakes, Fairbank and Windy (32 and 35 km from Sudbury centre, respectively), were part of both large and small genotype clades and the large standard deviation of the morphological analysis of Windy Lake specimens suggests there may be a combination of small and large body sizes. The laboratory specimens reared from a historic southern Ontario location grouped with the large genotype of the inner specimens. Our morphological analyses corroborate the molecular results by demonstrating that specimens collected within 30 km of the city centre constitute a different size group, significantly larger, than the specimens collected from more distant lakes.

Although the work presented here does not examine the fitness of the different genotypes or their adaptations, if such adaptations to different environments exist, it is interesting to speculate on possible differences between the genotypes based on previous work. In other locations, large-bodied *Hyalella* have been associated with lakes where fish predation is reduced or absent (Strong 1972; Wellborn et al. 2005), and the disturbance and contamination history of Sudbury has resulted in many lakes with damaged fish communities (i.e., reduced species richness, altered size distributions, and habitat use). Size-selective predation may maintain the small-body ecomorph (Wellborn and Broughton 2008); however, it does not necessarily explain why the large-body ecomorph is the colonizing group of the inner city lakes. Amphipods do not have strong dispersal capabilities and often rely on passive dispersal (Peck 1975; Witt and Hebert 2000; Bilton et al. 2001; Stutz et al. 2010). Physical adaptations, such as spines or spurs, may therefore play an important role in their dispersal ability. Although studies have reported that small-bodied amphipods may disperse more readily than larger individuals (Franz and Mohamed 1989), the presence of a dorsal spine (mucronation) may also aid in attachment for passive dispersal (Bilton et al. 2001). Size-adjusted spine length of *Hyalella* has been examined between genotypes showing the large morphotype had longer dorsal spines than the smaller morphotype where the spines may even be lacking (Wellborn 1995; Wellborn et al. 2005). Spine length was not examined for this study but may help in future morphological analyses of this group, possibly increasing our understanding of dispersal theories for these taxa.

Differences in traits, or genes, can also have significant impact on populations' tolerance to stressed or toxic environments. Studies on differential susceptibility to toxicity by different genotypes of *H. azteca* have been inconclusive for zinc (Duan et al. 1997) but have shown significant differences in resistance for polycyclic aromatic hydrocarbons (PAHs), pH, and other metals (Duan et al. 2000*a*, 2000*b*). Such differential susceptibility can affect the frequency and distribution of genotypes in wild populations, especially those recovering from severe contamination. Future work on the Sudbury populations will examine the toxicological sensitivities of these amphipods and determine if other physical or chemical habitat characteristics may be limiting the colonization of small-bodied genotype.

Finally, the sequence divergence (19.2%–26.7%) found between clades is consistent with past studies of *Hyalella* in North America showing nucleotide sequence divergence in the ranges of 9%–28% (Witt and Hebert 2000), 4%–30% (Witt et al. 2006), and 16%–20% (Wellborn and Broughton 2008). In the past, the threshold of 3% sequence divergence has been used to delineate species through molecular techniques (Hebert et al. 2003); however, a number of studies have found much higher sequence variation in otherwise similar individuals (Cognato 2006; Leo et al. 2010) or those from successful breeding trials (Lysyk and Scoles 2008). Although further studies of morphological and molecular differences are needed, we support the assertion that the *Hyalella* species complex may contain numerous species and may reach over 30 species (Witt et al. 2006; G.A. Wellborn personal communication).

In conclusion, the genetic and morphological analyses show *Hyalella* populations reestablishing in the industrially damaged region of Sudbury have a distinct genotype and are significantly larger than amphipods collected from lakes farther away from the city. Although a number of theories for the distribution pattern of these ecomorphs have been discussed here, ultimately, long-term monitoring of *Hyalella* reestablishment into the Sudbury lakes and laboratory experiments will aid in the understanding of the species toxicological sensitivities, habitat requirements, and dispersal capabilities.

Acknowledgements

We thank members of the Freshwater Cooperative Ecology Unit, Sudbury, Ontario, for their assistance with amphipod sampling and we are grateful to G. Wellborn for his useful advice and suggestions about morphological measurements. We also thank staff at the Canadian Centre for DNA Barcoding (CCDB) for their assistance in acquiring DNA barcodes from specimens. Funding for this project was supplied by the City of Greater Sudbury, and the Natural Sciences and Engineering Research Council of Canada (NSERC) through Discovery Grants (T.M., J.G., and D.L.), Canada Research Chair Grants (T.M. and J.G.), and a Collaborative Research and Development Grant (J.G.) with Vale Ltd. and Xstrata Ltd. as industrial partners.

References

Bilton, D.T., Freeland, J.R., and Okamura, B. 2001. Dispersal in freshwater invertebrates. Annu. Rev. Ecol. Syst. **32**(1): 159–181. doi:10.1146/annurev.ecolsys.32.081501.114016.

Cognato, A.I. 2006. Standard percent DNA sequence difference for insects does not predict species boundaries. J. Econ. Entomol. **99**(4): 1037–1045. doi:10.1603/0022-0493-99.4.1037. PMID: 16937653.

Duan, Y., Guttman, S.I., and Oris, J.T. 1997. Genetic differentiation among laboratory populations of *Hyalella azteca*: implications for toxicology. Environ. Toxicol. Chem. **16**(4): 691–695. doi:10.1002/etc.5620160413.

Duan, Y., Guttman, S.I., Oris, J.T., and Bailer, A.J. 2000*a*. Genotype and toxicity relationships among *Hyalella azteca*: I. Acute exposure to metals or low pH. Environ. Toxicol. Chem. **19**(5): 1414–1421. doi:10.1002/etc.5620190525.

Duan, Y., Guttman, S.I., Oris, J.T., Huang, X., and Burton, G.A. 2000*b*. Genotype and toxicity relationships among *Hyalella azteca*: II. Acute exposure to fluoranthene-contaminated sediment. Environ. Toxicol. Chem. **19**(5): 1422–1426. doi:10.1002/etc.5620190526.

Environment Canada. 1997. Biological test method: test for survival and growth in sediment using the freshwater amphipod *Hyalella azteca*. EPS 1/RM/33. Environment Canada, Ottawa, Ont.

Franz, D.R., and Mohamed, Y. 1989. Short-distance dispersal in a fouling community amphipod crustacean, *Jassa marmorata* Holmes. J. Exp. Mar. Biol. Ecol. **133**(1–2): 1–13. doi:10.1016/0022-0981(89)90154-8.

Hebert, P.D.N., Ratnasingham, S., and de Waard, J.R. 2003. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. Sci. **270**(Suppl. 1): S96–S99. doi:10.1098/rsbl.2003.0025. PMID:12952648.

Hogg, I.D., Larose, C., Lafontaine, Y.d., and Doe, K.G. 1998. Genetic evidence for a *Hyalella* species complex within the Great Lakes – St. Lawrence River drainage basin: implications for ecotoxicology and conservation biology. Can. J. Zool. **76**(6): 1134–1152. doi:10.1139/z98-034.

Huelsenbeck, J.P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, **17**(8): 754–755. doi:10.1093/bioinformatics/17.8.754. PMID:11524383.

Ivanova, N.V., and Grainger, C.M. 2007*a*. CCDB protocols, COI amplification. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification.pdf [accessed 15 July 2010].

Ivanova, N.V., and Grainger, C.M. 2007*b*. CCDB protocols, sequencing. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Sequencing.pdf [accessed 27 July 2010].

Ivanova, N.V., deWaard, J.R., and Hebert, P.D.N. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Mol. Ecol. Notes, **6**(4): 998–1002. doi:10.1111/j.1471-8286.2006.01428.x.

Ivanova, N.V., DeWaard, J.R., and Hebert, P.D. 2007. CCDB protocols, glass fiber plate DNA extraction. Available from http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_DNA_Extraction.pdf [accessed 15 July 2010].

Keller, W., Yan, N.D., Gunn, J.M., and Heneberry, J. 2007. Recovery of acidified lakes: lessons from Sudbury, Ontario, Canada. Water Air Soil Pollut. **7**(1–3): 317–322. doi:10.1007/s11267-006-9061-2.

Kozlov, M.V., and Zvereva, E.L. 2007. Industrial barrens: extreme habitats created by non-ferrous metallurgy. Rev. Environ. Sci. Biotechnol. **6**(1–3): 231–259. doi:10.1007/s11157-006-9117-9.

Leo, S.S.T., Pybus, M.J., and Sperling, F.A.H. 2010. Deep mitochondrial DNA lineage divergences within Alberta populations of *Dermacentor albipictus* (Acari: Ixodidae) do not indicate distinct species. J. Med. Entomol. **47**(4): 565–574. doi:10.1603/ME10006. PMID:20695271.

Lysyk, T.J., and Scoles, G.A. 2008. Reproductive compatibility of prairie and montane populations of *Dermacentor andersoni*. J. Med. Entomol. **45**(6): 1064–1070. doi:10.1603/0022-2585(2008)45[1064:RCOPAM]2.0.CO;2. PMID:19058630.

Mackauer, M. 1976. Genetic problems in the production of biological control agents. Annu. Rev. Entomol. **21**(1): 369–385. doi:10.1146/annurev.en.21.010176.002101.

Neary, B.P., Dillon, P.J., Munro, J.R., and Clark, B.J. 1990. The acidification of Ontario lakes: an assessment of their sensitivity and current status with respect to biological damage. Ontario Ministry of Environment, Dorset.

Peck, S.B. 1975. Amphipod dispersal in the fur of aquatic mammals. Can. Field-Nat. **89**: 181–182.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. **25**(7): 1253–1256. doi:10.1093/molbev/msn083. PMID:18397919.

Potvin, R., and Negusanti, J. 1995. Declining industrial emissions, improving air quality and reduced damage to vegetation. *In* Environmental restoration and recovery of an industrial region. *Edited by* J. Gunn. Springer-Verlag, New York. pp. 51–66.

Ratnasingham, S., and Hebert, P.D.N. 2007. BOLD: The Barcode of Life Data system (http://www.barcodinglife.org. Mol. Ecol. Notes, **7**(3): 355–364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:18784790.

Stamatakis, A., Hoover, P., and Rougemont, J. 2008. A rapid bootstrap algorithm for the RAxML web servers. Syst. Biol. **57**(5): 758–771. doi:10.1080/10635150802429642. PMID:18853362.

Strong, D.R. 1972. Life history variation among populations of an amphipod (*Hyalella azteca*). Ecology, **53**(6): 1103–1111. doi:10.2307/1935422.

Stutz, H.L., Shiozawa, D.K., and Evans, R.P. 2010. Inferring dispersal of aquatic invertebrates from genetic variation: a comparative study of an amphipod and mayfly in Great Basin springs. J. N. Am. Benthol. Soc. **29**(3): 1132–1147. doi:10.1899/09-157.1.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24**(8): 1596–1599. doi:10.1093/molbev/msm092. PMID:17488738.

Tao, J., Ingersoll, C., Kemble, N., Dias, J., Murowchick, J., Welker, G., and Huggins, D. 2010. Sediment contamination of residential streams in the metropolitan Kansas City Area, USA: Part II. Whole-sediment toxicity to the amphipod *Hyalella azteca*. Arch. Environ. Contam. Toxicol. **59**(3): 370–381. doi:10.1007/s00244-010-9498-1. PMID:20396875.

USEPA. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. U.S. Environmental Protection Agency (USEPA), Washington, D.C. EPA 600/R-99/064.

Wellborn, G.A. 1995. Predator community composition and patterns of variation in life history and morphology among *Hyalella* (Amphipoda) populations in southeast Michigan. Am. Midl. Nat. **133**(2): 322–332. doi:10.2307/2426397.

Wellborn, G.A., and Broughton, R.E. 2008. Diversification on an ecologically constrained adaptive landscape. Mol. Ecol. **17**(12): 2927–2936. doi:10.1111/j.1365-294X.2008.03805.x. PMID:18522695.

Wellborn, G.A., Cothran, R., and Bartholf, S. 2005. Life history and allozyme diversification in regional ecomorphs of the *Hyalella azteca* (Crustacea: Amphipoda) species complex. Biol. J. Linn. Soc. **84**(2): 161–175. doi:10.1111/j.1095-8312.2005.00422.x.

Wesolek, B.E., Genrich, E.K., Gunn, J.M., and Somers, K.M. 2010. Use of littoral benthic invertebrates to assess factors affecting biological recovery of acid- and metal-damaged lakes. J. N. Am. Benthol. Soc. **29**(2): 572–585. doi:10.1899/09-123.1.

Witt, J.D.S., and Hebert, P.D.N. 2000. Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. Can. J. Fish. Aquat. Sci. **57**(4): 687–698. doi:10.1139/f99-285.

Witt, J.D.S., Threloff, D.L., and Hebert, P.D.N. 2006. DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. Mol. Ecol. **15**(10): 3073–3082. doi:10.1111/j.1365-294X.2006.02999.x. PMID:16911222.