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Daniel J. Ryan
Purdue University

Maria S. Sepúlveda
Purdue University

Thomas F. Nalepa
National Oceanic and Atmospheric Administration, thomas.nalepa@noaa.gov

Tomas O. Höök
Purdue University, thook@purdue.edu

Spatial variation in RNA:DNA ratios of *Diporeia* spp. in the Great Lakes region

Daniel J. Ryan [a], Maria S. Sepúlveda [a], Thomas F. Nalepa [b], Tomas O. Höök [a],[*]

[a] *Purdue University, Department of Forestry and Natural Resources, 715 West State Street, West Lafayette, IN 47907, USA*
[b] *National Oceanic and Atmospheric Association, Great Lakes Environmental Research Laboratory, 4840 S. State Road, Ann Arbor, MI 48108, USA*

A B S T R A C T

Over the past two decades, *Diporeia* in all of the Laurentian Great Lakes, except Superior, have declined dramatically. These declines have seemingly coincided with expansion of invasive *Dreissena polymorpha* and *D. bugensis*, however the exact mechanisms underlying decreasing *Diporeia* densities are obscure. We explored the use of RNA:DNA (R/D) ratios as a conditional index for *Diporeia* by experimentally demonstrating that *Diporeia* R/D responds to periods of starvation. Moreover, during 2008–2009 we collected *Diporeia* from throughout the Great Lakes and Cayuga Lake (New York, USA), and used R/D ratios to index condition of these *in situ* collected animals. We evaluated spatial and temporal variation of nucleic acid indices using classification and regression tree (CART) analysis with a suite of environmental variables included as potential predictors. *Diporeia* R/D of *in situ* collected specimens exhibited pronounced spatial and temporal variation, but multiple CART models described only a small amount of this variation. While we observed some variation in *Diporeia* R/D among lakes, nucleic acid ratios appeared to respond weakly to *Diporeia* population characteristics and local environmental conditions. Specifically, CART analyses revealed that *Diporeia* R/D was particularly low at extreme depths, and interestingly, *Diporeia* nucleic acids were not strongly associated with the presence of dreissenids. In summary, while a limited amount of variation in *Diporeia* R/D was attributable to environmental conditions, the majority of *Diporeia* R/D variation was unaccounted for. Hence, the causative factors underlying spatio-temporal variation of *Diporeia* R/D and the mechanistic reasons for *Diporeia* declines in the Great Lakes remain largely unknown.

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Introduction

A widespread ecological shift in benthic communities of the Great Lakes region has occurred within the past two decades. The once abundant, deep water genus of amphipods, *Diporeia* spp. (hereafter, referred to as *Diporeia*), has declined precipitously in lakes Erie, Huron, Michigan and Ontario. While the underlying mechanisms of *Diporeia* collapse are poorly understood, it is evident that *Diporeia* declines have resulted in a suite of ecological consequences. *Diporeia* is a detritivore that feeds at the sediment–water interface, and recycles pelagic and benthic nutrients to higher trophic levels (Flint, 1986; Quigley and Vanderploeg, 1991; Fitzgerald and Gardner, 1993; Nalepa et al., 2006b). In fact, declines in lipid rich *Diporeia* have been linked to fish malnutrition resulting in reduced overall fish growth rate, condition and delayed maturation, especially for economically important lake whitefish (*Coregonus clupeaformis*) (Pothoven et al., 2001; Hondorp et al., 2005; Hoyle, 2005; Pothoven, 2005; Wang et al., 2008).

Spatial patterns of *Diporeia* declines across the Great Lakes region are quite complex, but have been hypothesized to be influenced by the expansion of non-native *Dreissena* spp. (*D. polymorpha*, and in particular, *D. bugensis*) populations. In Lake Erie, *Diporeia* have been seemingly extirpated after dreissenid establishment (Dermott and Kerec, 1997). Vast areas once inhabited by *Diporeia* in lakes Huron, Michigan and Ontario are now devoid of the amphipod, and some extirpations have occurred in areas far removed from dreissenids (Dermott et al., 2005b; Nalepa et al., 2006b; Watkins et al., 2007). *Diporeia* populations in these lakes now persist in deep areas, where dreissenid impacts are thought to be low (Dermott et al., 2005b). In Lake Superior, dreissenid populations are limited to shallow productive bays (Grigorovich et al., 2003, 2008) and *Diporeia* populations appear to be stable (Scharold et al., 2004; Auer et al., 2009). Batchawanna Bay, Lake Superior, is noteworthy in that this relatively shallow embayment is devoid of dreissenids and has extant *Diporeia* populations (Dermott, 1995). However, not all *Diporeia* populations that coexist with dreissenids are declining. For example, Cayuga Lake, a Finger Lake in New York State, has large co-existing populations of both taxa (Dermott et al., 2005a).

Spatial variation in *Diporeia* condition may be affected by various environmental factors. For instance, *Diporeia* condition in profundal areas of the Great Lakes may be limited by the quantity and quality of organic matter inputs to extreme depths (Evans et al., 1990; Meyers and Eadie, 1993). Potentially contributing to this phenomenon, dreissenid populations upslope of *Diporeia* populations may

limit diatoms from settling in deep locations (Guiguer and Barton, 2002). Location is known to influence amphipod fecundity as well as condition, especially since organic matter availability in a specific lake is largely dependent on lake trophic status (Sundelin et al., 2008). Competitive grazing between individual *Diporeia* may alter food availability, thereby influencing individual *Diporeia* condition. Lastly, seasonal variations in food availability may affect *Diporeia* condition given that *Diporeia* growth is highly dependent on spring diatom blooms (Gardner et al., 1985; Fitzgerald and Gardner, 1993).

Given the complexities of *Diporeia* declines, analysis of spatial variation of *Diporeia* condition may contribute to elucidating mechanisms underlying declines. Using *a priori* knowledge of extant *Diporeia* populations, we investigated spatial variation of nucleic acid concentrations (RNA and DNA) in *Diporeia*. RNA:DNA (R/D) ratios are a short-term indicator of condition and have been quantified for a variety of organisms (Buckley, 1984; Bulow, 1987; Speekmann et al., 2007; Johannsson et al., 2008; MacLean et al., 2008), including *Diporeia* (Kainz et al., 2010). Within a cell, RNA is transcribed to initiate protein synthesis while DNA concentrations remain fairly constant (Bulow, 1987; Dahlberg, 1989). Thus, relatively high R/D values indicate an actively growing organism while low ratios are indicative of an organism exhibiting little growth. Past studies on a variety of organisms have indicated that nucleic acid ratios respond to organism size as well as multiple environmental factors, such as temperature and food availability (Buckley et al., 1984; Wagner et al., 2001; Johannsson et al., 2008; Schlechtriem et al., 2008). Ontogenic variation in DNA concentrations of invertebrates could imply that whole body RNA concentrations are more sensitive indices of condition than R/D (*e.g.*, Gorokhova, 2003; Holmborn et al. 2009). However, Ryan (2010) quantified both total RNA content and R/D ratios of experimental and *in situ* collected *Diporeia*, and found that these two indices revealed very similar patterns. Moreover, Kainz et al. (2010) recently determined that *Diporeia* R/D ratios respond to food quality, thereby providing precedence for the use of R/D ratios to assess *Diporeia* condition.

While studies of diverse taxonomic groups demonstrate that nucleic acid ratios respond to various environmental factors (*e.g.*, food, temperature) and index short-term growth (Buckley et al.,

1984; Bulow, 1987; Clemmesen, 1994; Wagner et al., 1998; Vrede et al., 2002; Schlechtriem et al., 2008), only a limited number of studies have explored the response of R/D ratios to such factors in amphipods, including *Diporeia* (Sutcliffe, 1965; Wu and Or, 2005; Kainz et al., 2010). We conducted experiments to independently evaluate the usefulness of RNA:DNA as an index for *Diporeia* condition. Moreover, we examined the variability of R/D ratios in *Diporeia* collected across the Great Lakes region and over different temporal periods (years, seasons). We hypothesized that 1) *Diporeia* R/D values would respond negatively to periods of starvation during experimentation and 2) R/D values of *in situ* collected *Diporeia* would be relatively high during spring and in shallow water areas, and be relatively low in areas where dreissenids and *Diporeia* co-exist.

Methods

Diporeia collections

During spring, summer and fall of 2008 and 2009, *Diporeia* were collected from multiple sites in lakes Huron, Michigan, Ontario, Superior and Cayuga (New York, USA) (Fig. 1 and Supplemental Table 1). Sites in the Great Lakes were the same as those routinely monitored by the Environmental Protection Agency-Great Lakes National Program Office (EPA-GLNPO), or those within the benthic monitoring program of the National Oceanic and Atmospheric Association-Great Lakes Environmental Research Laboratory (NOAA-GLERL) (Nalepa et al. 2006a). Within both monitoring programs, sites were selected primarily based on historical *Diporeia* abundances and secondarily based on dreissenid presence or absence. Moreover, the number of *Diporeia* present and weather conditions at the time of collection were limiting factors in determining which sites were sampled.

Diporeia were collected from the Great Lakes with a Ponar grab (0.23 × 0.23 m opening with 500 μm mesh) and from Cayuga Lake with a Petite Ponar grab (0.15 × 0.15 m opening with 500 μm mesh). Time permitting, up to 3 grabs were washed through a 500 μm screen and retained material was preserved in 10% formalin containing rose bengal stain. These samples were used to estimate abundances of
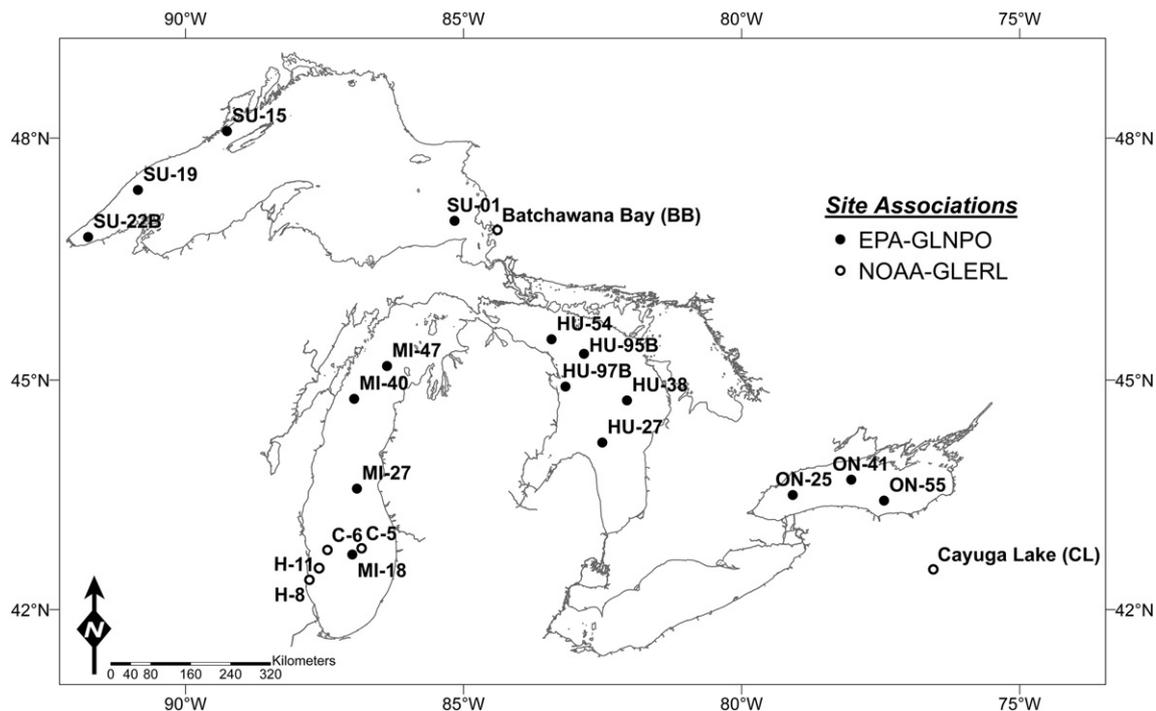

Fig. 1. Benthic sites in the Great Lakes region sampled during 2008 and 2009. Closed and open circles depict EPA-GLNPO and NOAA-GLERL sites, respectively.

Diporeia and dreissenids. In the laboratory, *Diporeia* and dreissenids in these formalin preserved samples were sorted in glass pans under a magnification lens (1.5×) and invertebrate densities were determined as no./m². *D. polymorpha* co-occurred with *D. bugensis* in some samples, but comprised at most 2% of dreissenids collected at any location, hence these data were combined as total dreissenid density.

Animals for a starvation experiment were collected on day of year (DOY) 270, 2009 from site C-6 in Lake Michigan (Fig. 1). This site was chosen because it had relatively high densities of *Diporeia* based on previous sampling and was relatively proximate to our laboratory. Upon collection, *Diporeia* were gently removed from benthic samples and placed in containers with 4 °C lake water. In addition, a subset (n = 42) of *Diporeia* was immediately preserved in 1.0 mL of RNA*later*® to compare *in situ* R/D values to experimental R/D values. Containers were kept at 4 °C in the dark and transported to Purdue University (West Lafayette, IN), where they were held overnight (darkness, 4 °C) prior to the start of the experiment.

In addition, extra Ponar grabs were taken at each site to obtain *in situ Diporeia* samples for RNA and DNA quantification. Upon collection, live animals were gently screened from the sediments and individuals immediately placed in vials with 1.0 mL RNA*later*®, a supersaturated salt solution that preserves nucleic acids. Several past studies have successfully relied on RNA*later*® to preserve nucleic acids of various experimental and *in situ* collected animals (Gorokhova and Kyle, 2002; Gorokhova, 2003; Johnson et al., 2003; Höök et al., 2008).

Starvation experiment

Before the start of the experiment, a subset of *Diporeia* was preserved in RNA*later*® (n = 29) to quantify baseline, pre-experiment nucleic acid concentrations after animals were exposed to potential stressors associated with collection and transport to the laboratory. Remaining *Diporeia* (n = 125) were placed individually into mesocosms that were constructed with 125 mL Erlenmeyer flasks. Each flask contained approximately 85 g of sieved and autoclaved Lake Michigan sand as a substrate for burrowing, 75 mL of Lake Michigan water collected at a depth of 100 m and one *Diporeia*. Flasks were kept in darkness at 4 °C inside a walk-in cooler. After 15 experimental days, water in flasks was exchanged by decanting approximately half the original volume and refilling with new Lake Michigan water. Random samples of 40 *Diporeia* were removed on days 3, 15 and 30 from the start of the experiment (5 additional experimental animals were allotted to account for losses due to mortality). Live *Diporeia* were immediately preserved in 1.5 mL microcentrifuge vials containing 1.0 mL of RNA*later*®, and stored at 4 °C (Gorokhova, 2005). Equipment used was routinely cleaned with RNase-OFF™ to prevent contamination of samples.

Determination of nucleic acids

Individuals from the starvation experiment and individuals preserved immediately in the field were removed from RNA*later*®, suspended laterally in RNase and DNase free water, lengths measured as described previously and adjusted for preservation effects (Abramoff et al., 2004; Foley et al., 2010). Forceps and Petri dishes used in length measurements were routinely cleaned with RNase-OFF™ to prevent contamination.

After length measurements, *Diporeia* were placed in 1.0 mL of extraction buffer for extraction of nucleic acids. Whole *Diporeia* were homogenized mechanically with five grinding motions from an RNA-ase free pestle, followed by a triplicate sequence of 30 second ultrasonic treatment (Cole-Parmer, model# 08849-00) and 1 min intermittent ice bath. Samples were then placed on an orbital shaker for 2 h. Approximately 1% of the organisms' nucleic acids were removed for fluorometric determination (2 subsamples at 5 μL/

subsample). RNA and DNA standards and negative controls were prepared and analyzed in duplicate, concurrent with duplicate *Diporeia* samples. Samples were placed in a 96-well, bottom rounded microplate (Costar 3915), 70 μL of the fluorophore RiboGreen® was added to all wells and total fluorescence was measured in a FLx800 multi-detection microplate reader (BioTek Instruments, Inc.). Then, 5 μL of endoribonuclease RNase was added to all wells, samples were incubated at approximately 37.5 °C for 30 min (temperature corresponding to maximum RNase enzyme efficiency) and total fluorescence was again measured. Gen5™ (BioTek Instruments, Inc.) microplate data collection and analysis software was used to quantify total sample fluorescence. RNA and DNA concentrations were estimated through comparison with standard curve fluorescence and RNA and DNA (μg/*Diporeia*) and R/D ratios were determined. We standardized all experimental and *in situ* collected samples to the mean standard curve slope ratio (2.44) (Berdalet et al., 2005; Caldarone et al., 2006). For specific reagents and reagent dilutions see Ryan (2010).

Data analysis: starvation experiment

Since individuals of different sizes were used in the starvation experiment, we evaluated allometric effects on nucleic acid expression as appropriate and accounted for size effects. To this end, linear regressions were used to determine if R/D ratios varied significantly with *Diporeia* length. If this relationship was not significant, an analysis of variance (ANOVA) and Tukey post-hoc test were used to compare between *in situ* specimens, day 0, day 3, day 15 and day 30 treatments. All statistical analyses were performed in SPSS software (version 16.0.2) and significance evaluated at α = 0.05.

Data analysis: in situ collected Diporeia

Given that the nucleic acid patterns of *Diporeia* likely respond to a suite of factors, with *a priori* undefined relationships, we used classification and regression tree analysis (CART) as an exploratory statistical approach to evaluate spatial, temporal, environmental and density effects on *Diporeia* nucleic acid patterns. Analysis of spatio-temporally variable environmental data can be complicated, given the potential absence of *a priori* model structure and complexities associated with both continuous and categorical predictive data. CART analysis is a simple, easily interpretable and increasingly used non-parametric statistical technique (Breiman et al., 1984; Lamon and Stow, 1999; Rejwan et al., 1999; De'ath and Fabricius,

Table 1
Variables used in classification and regression tree analyses to model *Diporeia* RNA: DNA (R/D) ratios. Pooled agency models represent spring and summer data combined from EPA-GLNPO and NOAA-GLERL sites, while NOAA-GLERL models include data from NOAA-GLERL sites collected during spring, summer and fall. Abundance was averaged over the spring and summer for pooled agency models, but not averaged for NOAA-GLERL models. C = categorical data, N = continuous data.

Variables	Type	Values
Pooled data models		
Season	C	Spring, summer
Lake	C	Huron, Michigan, Ontario, Superior, Cayuga
Dreissena	C	False (absent), true (present)
Depth	N	Depth (m)
Length	N	>3 mm
Abundance	N	Mean (no./m²) for spring or spring and summer collections
NOAA-GLERL models		
Season	C	Spring, summer, fall
Lake	C	Cayuga, Michigan, Superior
Dreissena	C	False (absent), true (present)
Depth	N	Depth (m)
Length	N	>3 mm
Abundance	N	No./m²

Table 2
Density estimates (no./m^{-2} ± SE) for *Diporeia* and dreissenids and mean RNA:DNA (R/D ± SE) of *Diporeia* during spring, summer and fall of 2008 and 2009. Mean R/D ratios for *Diporeia* are presented where available. Crosses denote absence of targeted organism and asterisks denote presence of targeted organism, but values were not quantified. Blanks indicate that the site was not sampled during a time period.

Lake/Site	Season	2008			2009		
		Diporeia density (No./m^2)	*Dreissena* spp. density (No./m^2)	Average *Diporeia* RNA:DNA	*Diporeia* density (No./m^2)	*Dreissena* spp. density (No./m^2)	Average *Diporeia* RNA:DNA
Cayuga Lake							
CL	Spring	2112 ± 734	17,398 ± 7171	0.68 ± 0.06	3477 ± 1562	11,580 ± 1011	0.57 ± 0.03
	Summer	3764 ± 899	14,726 ± 5290	0.38 ± 0.05	2428 ± 517	10,761 ± 1628	0.62 ± 0.03
	Fall	3534	*	0.34 ± 0.03	6997 ± 791	11,321 ± 4844	0.84 ± 0.07
Lake Huron							
HU-27	Spring	†	†	†			
	Summer	†	†	†			
HU-38	Spring	*	†	*	149 ± 21	†	0.58 ± 0.03
	Summer	*	†	*	*	†	0.66 ± 0.05
HU-54	Spring	979 ± 128	*	0.7 ± 0.06	*	*	0.51 ± 0.03
	Summer	*	*	0.33 ± 0.03	*	*	0.73 ± 0.04
HU-95B	Summer	*	†	*	*	†	*
HU-97B	Spring	†	†	†			
Lake Michigan							
MI-18	Spring	340 ± 191	†	0.3 ± 0.05	787 ± 21	†	0.93 ± 0.03
	Summer	*	†	0.15 ± 0.02	*	†	0.33 ± 0.03
MI-27	Summer	†	*	†			
MI-40	Spring	128	†	0.78 ± 0.11	383 ± 319	†	0.47 ± 0.04
	Summer	*	*	*	*	†	0.75 ± 0.03
MI-47	Summer	*	†	*			
C-5	Spring	745 ± 43	†	0.42 ± 0.04	149 ± 33	*	*
	Summer	766 ± 21	*	0.48 ± 0.04			
	Fall	*	†	0.69 ± 0.04			
C-6	Spring				1007 ± 63	*	0.97 ± 0.09
	Summer				931 ± 84	1064	0.76 ± 0.05
	Fall				601 ± 92	1596	0.96 ± 0.07
H-8	Spring	†	†	†			
H-11	Spring	610 ± 138	4631 ± 2115	0.42 ± 0.04	816 ± 156	†	
	Summer	1287 ± 117	138 ± 96	0.43 ± 0.03	723 ± 140	362	0.69 ± 0.05
Lake Ontario							
ON-25	Spring	223 ± 32	*	0.38 ± 0.11	378 ± 147	234	0.98 ± 0.09
	Summer	*	†	*	223 ± 53	†	0.6 ± 0.02
ON-41	Spring	638 ± 85	*	0.49 ± 0.07	128	*	0.57 ± 0.05
	Summer	*	†	0.34 ± 0.03	*	*	0.91 ± 0.07
ON-55	Spring	521 ± 181	*	0.31 ± 0.05	*	*	0.44 ± 0.03
	Summer	*	†	0.34 ± 0.03	*	*	0.54 ± 0.12
Lake Superior							
SU-01	Spring	596 ± 43	†	0.43 ± 0.04	628 ± 11	†	0.52 ± 0.04
	Summer	*	†	0.49 ± 0.04	*	†	0.39 ± 0.04
SU-15	Spring	755 ± 287	†	0.19 ± 0.05	*	†	0.92 ± 0.28
SU-19	Spring				53 ± 11	†	0.56 ± 0.05
SU-22B	Summer	*	†	0.55 ± 0.06	*	†	0.76 ± 0.07
BB	Spring	213 ± 86	†	0.47 ± 0.06	206 ± 58	†	0.86 ± 0.04
	Summer	333 ± 28	†	1.03 ± 0.19	369 ± 47	†	0.75 ± 0.04
	Fall	596 ± 81	†	0.26 ± 0.05	369 ± 38	†	1.08 ± 0.05

2000). CART is advantageous over other multivariate techniques because it can use both categorical and numerical data to construct models, identify complex relationships and is an exploratory statistical technique used to facilitate understanding of complex ecological data (Breiman et al., 1984; Faraway, 2006). In short, the CART algorithm constructs a decision tree or dendogram by iteratively splitting the dataset based on predictor variables in order to minimize the within group variation of the response variable (Breiman et al., 1984; Faraway, 2006; Qian, 2010). Regression trees (Therneau et al., 2010) and corresponding boxplots of each terminal node were constructed in the statistical package R (R Development Core Team, 2009).

Data were excluded prior to performing CART to balance the statistical design. For instance, *Diporeia* abundances were low at some sites, and thus, only sites with n ≥ 18 *Diporeia* per sampling event were included (see Supplemental Table 1 for sites used in specific

models). Further, the length of *Diporeia* is known to be an indicator of ontogenic stage. *Diporeia* considered to be young of year (approximately ≤ 3 mm) were sampled inconsistently and excluded from regression trees (Winnell and White, 1984; Johnson, 1988; Nalepa et al., 2006a; Auer et al., 2009). Remaining data were evaluated for length effects using linear regression to verify that R/D ratios did not trend with *Diporeia* size. Four separate CART analyses were conducted, first by year (2008 and 2009) and then by agency within each year (EPA-GLNPO and NOAA-GLERL). Data were initially divided by year because sample sites varied between years, and *Diporeia* collected in 2008 were in RNA*later*© longer than their 2009 counterparts, potentially leading to varying amounts of *Diporeia* nucleic acids between years (Gorokhova, 2005). Next, data were divided by agency because some sites were sampled three times per year (NOAA-GLERL sites sampled spring, summer and fall, Table 1 and Supplemental Table 1), while other sites were only sampled twice per year (EPA-

GLNPO sites sampled spring and summer; Table 1 and Supplemental Table 1). Thus, yearly and agency samples were analyzed independently. While initial analyses did not reveal a linear association between *Diporeia* length and R/D ratios, organism length was nonetheless included as a potential explanatory variable to evaluate non-linear and higher order effects (*i.e.*, effects of length after accounting for influence of other explanatory variables). See Table 1 for a complete list of explanatory variables included in CART analyses.

The regression tree algorithm constructs a dendogram by iteratively partitioning dependent variable data into two groups (or nodes), whereby within group residual sum of squares for the response variable is minimized. Due to the hierarchical structure of the dendogram, initial splits generally lead to the greatest reductions in variation. For each analysis, an overly large tree was developed, reduced in size (*i.e.*, model pruning) through 10-fold cross-validation and this process repeated 100 times to build a distribution of trees where the modal tree was assumed to be a typical tree (De'ath and Fabricius, 2000). Selection of a conservative tree size was implemented by choosing the number of splits corresponding to the largest standard error within one standard error of the minimum cross-validation relative error (Breiman et al., 1984; De'ath and Fabricius, 2000; Faraway, 2006; Qian, 2010).

Results and discussion

Diporeia collections

During 2008 and 2009, densities of *Diporeia* and dreissenids were consistently higher in Cayuga Lake than other locations (Cayuga Lake total mean densities: $3719/m^2$) (Table 2), while *Diporeia* densities at other sample sites varied widely (range of total mean densities from other locations: $0/m^2$–$979/m^2$) (Table 2). Recent investigations indicate wide spatial variation in *Diporeia* abundances within and among the lakes investigated. Studies in lakes Huron, Michigan and Ontario have documented substantially reduced populations or complete absences of *Diporeia* (Dermott, 2001; Nalepa et al., 2006b, 2007, 2009), while collections in Lake Superior suggest that *Diporeia* remain stable (Scharold et al., 2004; Dermott et al., 2005a; Auer et al., 2009). In addition, densities of *Diporeia* in Cayuga Lake have remained high despite coexistence with prolific populations of dreissenids (Dermott et al., 2005a). Our density estimates presented herein provide an additional, updated assessment of *Diporeia* abundances in the Great Lakes region that are generally consistent with past descriptions.

Starvation experiment

Diporeia in the 3, 15 and 30 day treatments experienced high survival (80%), and we quantified nucleic acids for a large number of individuals (total: n = 100; 3d: n = 31, 15d: n = 34, 30d: n = 35). RNA and DNA for *Diporeia* exhibited significant relationships with individual length (RNA: p<0.0001, DNA: p<0.001, Fig. 2a) while R/D ratios did not (p = 0.34, Fig. 2b). We statistically compared R/D ratios of *in situ* preserved, transported (day 0) and starved (days 3, 15 and 30) *Diporeia*. The R/D ANOVA with Tukey post-hoc test suggested that the *in situ* specimens and 30 day treatment had a significantly higher and lower mean R/D ratio, respectively, than the 0, 3 and 15 day treatments ($F_{3, 125} = 14.51$, p<0.001, Fig. 3).

The starvation experiment was designed to independently evaluate R/D as an index for *Diporeia* condition by eliciting changes in *Diporeia* nucleic acids. Through this experiment, we observed a reduction in *Diporeia* R/D collected *in situ* and after a 30 day starvation period as expected. In addition, Ryan (2010) conducted various experiments manipulating feeding regime and temperature to examine the response of *Diporeia* nucleic acids. The experimental factors evaluated by Ryan (2010) in the laboratory did not always induce a short-term response in nucleic acid ratios. Slow metabolic rates at low
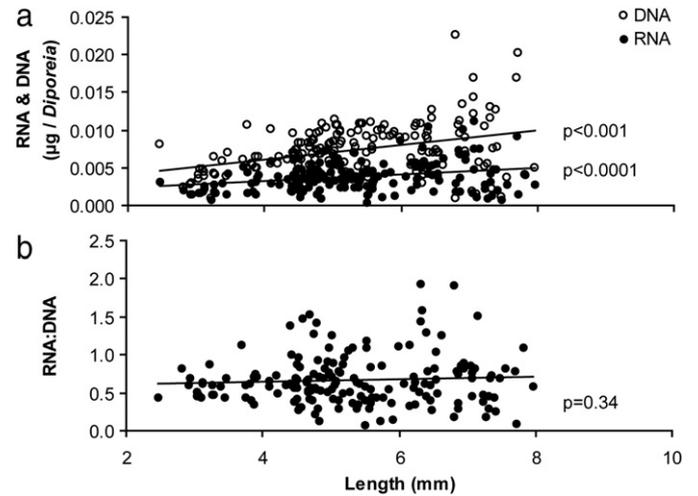

Fig. 2. *Diporeia* (a) RNA and DNA, and (b) RNA:DNA regressions with length from an experiment evaluating starvation effects.

temperatures likely delay the response of *Diporeia* to stressors, and ingestion of exuvia in the absence of food could delay starvation (Schlechtriem et al., 2008). *Diporeia* are known to feed intermittently and can seemingly endure periods of starvation (Quigley, 1988; Dermott and Corning, 1988, Gauvin et al. 1989, Evans et al., 1990), thus a relatively undisturbed 30 day duration of starvation may not be long enough to elicit large changes in *Diporeia* R/D. Moreover, *Diporeia* nucleic acid ratios are very low compared to other invertebrates (Wright and Hetzel, 1985; Chicharo and Chicharo, 1995; Schlechtriem et al., 2008). Kainz et al. (2010) found similar R/D values for *Diporeia*, however, Kainz et al.'s (2010) values are not directly comparable to our study without correcting for different standard curve slope ratios (Berdalet et al., 2005; Caldarone et al., 2006). Nonetheless, while it is not completely clear why *Diporeia* R/D values are extremely low in comparison to other invertebrates, these low ratios likely lead to reduced overall variation and could limit the ability to describe spatial patterns through CART analyses.

Diporeia nucleic acid ratios appear to be affected by severe short term stresses such as collection and transportation procedures. We observed a reduction of *Diporeia* R/D from collection in Lake Michigan to the start of experiments two days later (experimental day 0). Ryan (2010) observed similar short term reductions in R/D values during two other transportation events. Reduced *Diporeia* R/D ratios incurred through collection and transportation demonstrate that these indices respond to severe short term stresses (*e.g.*, changes in pressure, temperature and cessation of feeding). Moreover, this finding has implications for other researchers when they aim to interpret results from laboratory trials with field collected *Diporeia*.
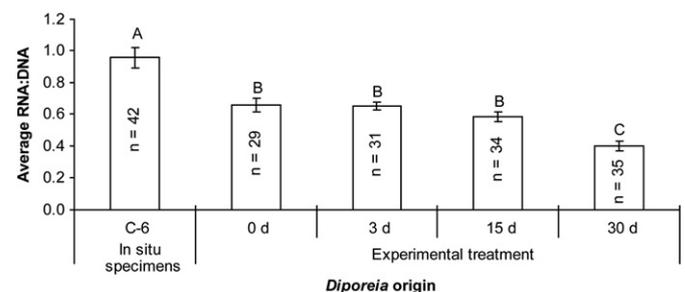

Fig. 3. Mean *Diporeia* RNA:DNA (R/D) for *in situ* preserved specimens and individuals starved for 0, 3, 15 and 30 days. Shared letters denote treatments without significant differences according to a Tukey post-hoc test and error bars depict standard errors.

In situ collected Diporeia

Diporeia R/D varied among collection sites and across seasons (Table 2). The greatest mean R/D, 0.89, was observed at site C-6 in Lake Michigan and the lowest mean R/D, 0.41, was observed at site ON-55 in Lake Ontario (intra-assay coefficient of variation: 0.097). Over the range of lengths evaluated with linear regression, we did not find any significant linear relationships between *Diporeia* R/D and individual organism length (Supplemental Fig. 1a–d). Nonetheless, we conservatively included individual length as a predictive variable in CART models to account for any higher order and non-linear relationship between *Diporeia* length and R/D.

Collectively, CART models explained a low amount of variation in *Diporeia* R/D. The regression trees developed using spring and summer R/D ratios for pooled models in 2008 and 2009 were depicted with one and seven splits, respectively (Fig. 4a–d). During 2008, depth was selected as the only predictive variable and explained a low amount of variation ($R^2 = 0.07$, $p < 0.0001$), while in 2009, depth and lake were selected as first and second splits and the model explained a greater amount of variation ($R^2 = 0.19$, $p < 0.0001$). *Diporeia* R/D from NOAA-GLERL sites during 2008 and

2009 were described with three and five splits, respectively (Fig. 5a–d). Initial splits for 2008 R/D data were based on abundance and season ($R^2 = 0.11$, $p < 0.0001$), with initial splits for 2009 based on season and lake ($R^2 = 0.18$, $p < 0.0001$). In the trees presented, the length of the vertical branches represents the variation explained by a split (Faraway, 2006; Qian, 2010).

Our measures of condition from *in situ* collected *Diporeia* were highly variable across space and time. Season, *Diporeia* length, abundance and depth (in decreasing order of appearance) appeared in CART analyses suggesting that these variables have minor influence on *Diporeia* condition. *Diporeia* condition would be expected to respond to spring diatom blooms, a historically important energy source (Gardner et al., 1985; Dermott and Corning, 1988; Quigley and Vanderploeg, 1991; Fitzgerald and Gardner, 1993). Nucleic acid indices varied seasonally, but spring peaks in *Diporeia* condition were not consistently reflected in dendograms. *Diporeia* length was selected in two dendograms as a higher order effect. The effect of length was both positive and negative, suggesting complex higher order, interactive effects of length. Abundance frequently appeared in regression trees, but effects of abundance on nucleic acid indices were not consistent. Low abundances could relate to 1) high *Diporeia*
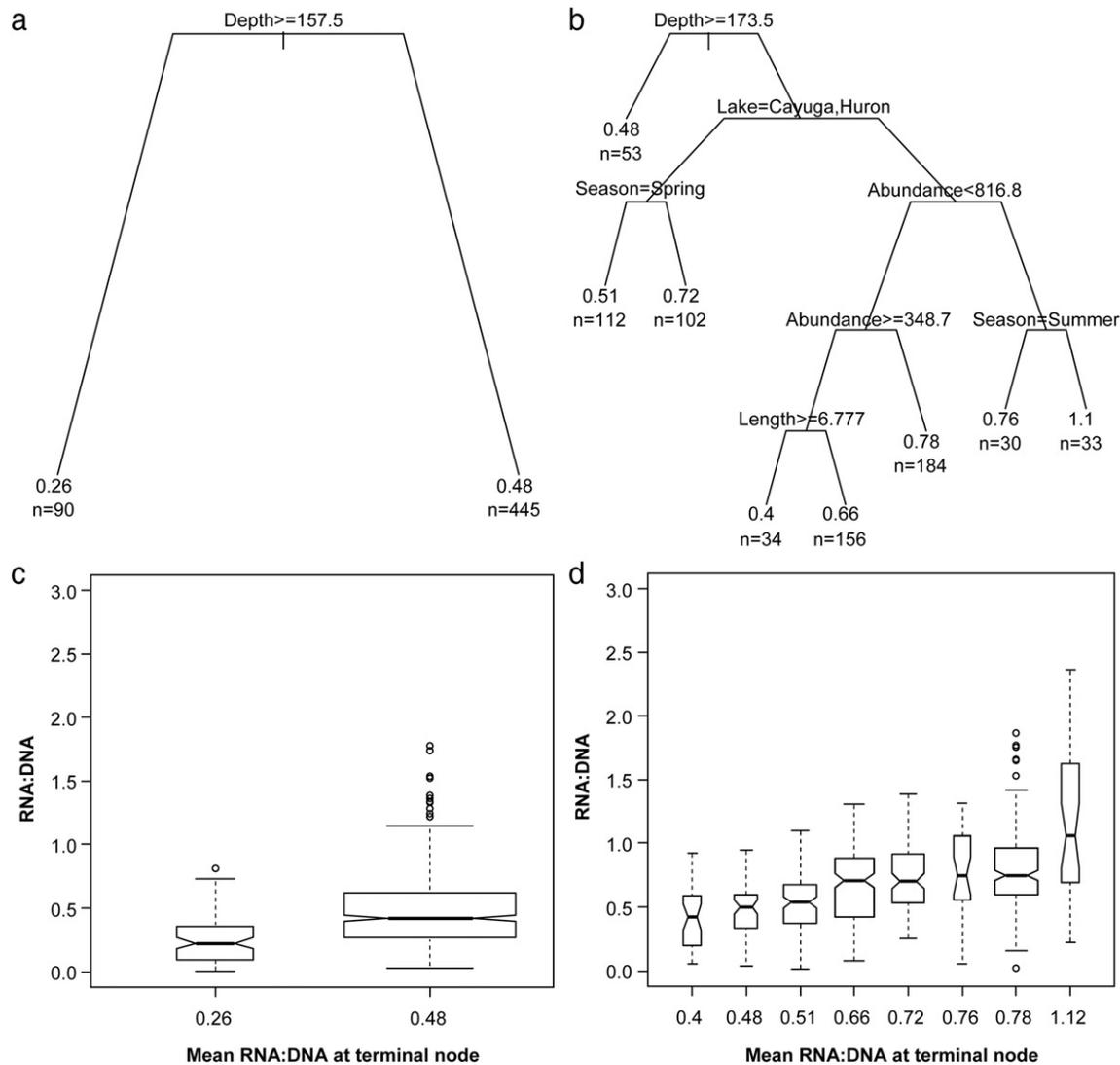
Fig. 4. Regression trees for (a) 2008 pooled and (b) 2009 pooled *Diporeia* RNA:DNA data. Data which adhere to the threshold presented at each node of the regression tree follow the left branch, while data which are counter to the threshold follow the right branch. Boxplots depict variation within terminal nodes of (c) 2008 and (d) 2009 regression trees. The width of each box in the boxplot is proportional to the square root of the number of samples in each terminal node. Notched boxes represent 95% confidence intervals about the median.
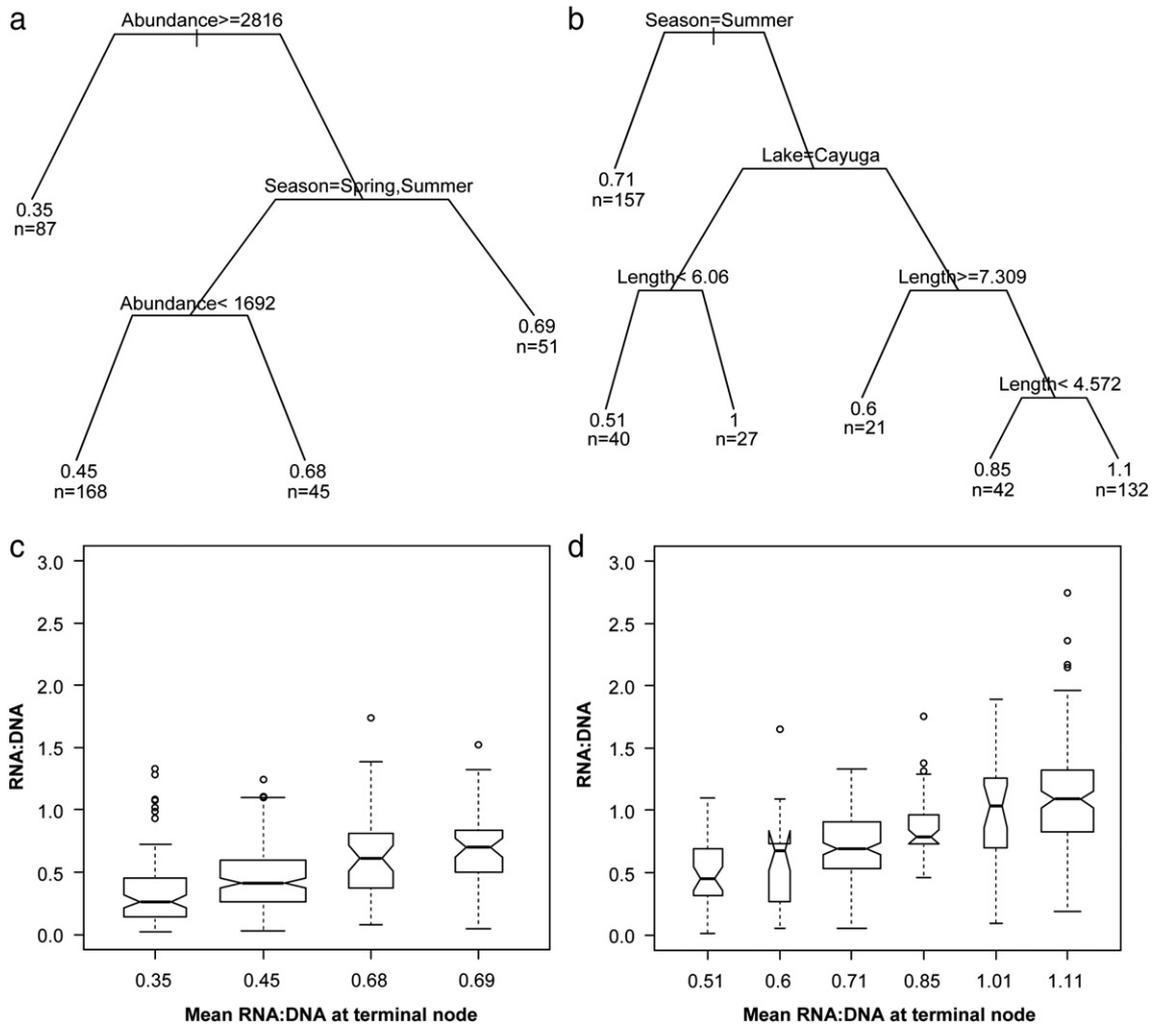
Fig. 5. Regression trees for (a) 2008 NOAA-GLERL and (b) 2009 NOAA-GLERL *Diporeia* RNA:DNA data. Data which adhere to the threshold presented at each node of the regression tree follow the left branch, while data which are counter to the threshold follow the right branch. Boxplots depict variation within terminal nodes of (c) 2008 and (d) 2009 regression trees. The width of each box in the boxplot is proportional to the square root of the number of samples in each terminal node. Notched boxes represent 95% confidence intervals about the median.

condition whereby survivors are released from density dependent processes (*i.e.*, an increase in food availability) or 2) low condition because *Diporeia* are responding to an unknown environmental stressor affecting both population density and individual condition.

Depth was selected as an initial split in multiple trees, suggesting that depth has a relatively moderate influence on *Diporeia* condition. CART analyses indicate marginally lower *Diporeia* condition at deeper sites. *Diporeia* in the Great Lakes appear to prefer slope regions over profundal regions (Evans et al., 1990; Auer et al., 2009), as growth, condition and survival may be low in deep, unproductive zones. Moreover, the potential dreissenid induced shunting of primary productivity to nearshore areas may have reduced detrital inputs to profundal areas (Guiguer and Barton, 2002; Hecky et al., 2004), and this could also reduce *Diporeia* condition. The combination of these factors suggests that *Diporeia* at deep sites exhibit lower condition.

The lake in which *Diporeia* were collected was selected as an intermediate splitting variable in determining *Diporeia* condition, suggesting that although *Diporeia* condition may vary somewhat among lakes, variation among individual sites is greater. This is not surprising, considering the large scale, spatially dynamic processes that occur within each Great Lake (*e.g.*, upwelling events, depositional zones) which can lead to dramatically different environmental conditions across a single lake (Boyce et al., 1989; Croley, 1992; Jude and

Leach, 1999; Nalepa et al., 2006b; Watkins et al., 2007). Several studies have attributed condition of invertebrates to similar dynamics (Gardner et al., 1985; Hill et al., 1992; Cavaletto et al., 1996; Nalepa et al., 2000), also suggesting that *Diporeia* R/D characteristics are more heavily influenced by site specific conditions rather than lake wide characteristics. It is also noteworthy that we observed no large across-lake differences in *Diporeia* R/D when comparing lakes where *Diporeia* populations are declining versus stable (*i.e.*, Lakes Michigan, Huron and Ontario v. Lakes Superior and Cayuga).

We did not find any associations between the presence of dreissenids and *Diporeia* R/D at individual sites. We simply evaluated the potential effect of presence or absence of dreissenids at a particular location, which may not have captured complex or remote interactions between the invertebrates. For instance, Watkins et al. (2007) suggested nearshore dreissenid activities remotely affect profundal *Diporeia* populations in Lake Ontario through competitive grazing and deposition of pseudofeces. Guiguer and Barton (2002) proposed that littoral epilithic algae plays an important role in the diets of offshore *Diporeia*, and thus, interception of this food source by dreissenids upslope could affect offshore *Diporeia* condition. Nonetheless, if dreissenids negatively impact *Diporeia*, this finding underscores the complex interactions between the two invertebrates (Dermott, 2001; Nalepa et al., 2006b; Watkins et al., 2007).

Conclusion

To explore spatial and temporal variation of *Diporeia* condition, we quantified nucleic acid indices and integrated measures from experiments and *in situ* collected *Diporeia*. Experimental results seem to suggest that nucleic acids are useful for indexing *Diporeia* condition in the laboratory, as found by Kainz et al. (2010). However, we observed a high degree of spatial and temporal R/D variation from *in situ Diporeia* and multiple CART analyses did not explain a large amount of this variation. Nonetheless, season, *Diporeia* length, abundance and depth contributed toward explaining a low amount of *Diporeia* R/D variation, while interestingly, the presence of dreissenids did not. The lack of strong relationships between environmental variables and *Diporeia* R/D is consistent with several other studies which have failed to find a consistent mechanism underlying declines of *Diporeia* in the Great Lakes.

The results of this study appear to have conflicting evidence on the usefulness of R/D to accurately index *Diporeia* condition. On one hand, experimental results that attempted to simulate hypolimnetic conditions demonstrate significant effects of starvation on R/D levels over intermediate time scales (14–30 days), and nucleic acid ratios seemingly respond to severe short term stresses such as handling and transport. Kainz et al. (2010) also demonstrated that R/D ratios of *Diporeia* respond to dietary content over 30 days. The independently evaluated results presented here, in conjunction with Kainz et al. (2010), indicate that *Diporeia* R/D can be influenced by controlled environments at intermediate time scales. In contrast, R/D for *in situ* collected *Diporeia* were highly variable both within and across sample sites, lacked strong associations with environmental conditions and comparisons between experimental and *in situ Diporeia* R/D were not strikingly different. It is plausible that the degree of ambient variability experienced by *Diporeia* at a specific location is far greater than the environmental variability observed by *Diporeia* across different locations within our study lakes. That is, since *Diporeia* inhabit cold, stable regions, their R/D values in the Great Lakes may not vary dramatically between the sites sampled. Moreover, intermittent feeding and ontogenic variation such as molting stage of *Diporeia* could lead to high individual variability in *Diporeia* R/D which could further confound ability to detect across site patterns (Quigley, 1988; Evans et al., 1990; Gorokhova and Kyle, 2002; Schlechtriem et al., 2008). Overall, it is known that R/D ratios can be successfully used to measure the condition of a variety of organisms, but the ability of R/D ratios to accurately index *Diporeia* condition for *in situ* collected specimens is poorly understood. Investigations into *Diporeia* physiology and how *Diporeia* nucleic acids are influenced by environmental conditions could potentially improve our understanding as to why spatial patterns in *Diporeia* R/D were highly variable. It remains an open question why *Diporeia* have declined precipitously in many areas of the Great Lakes. *Diporeia* R/D seemingly respond to feeding conditions (Kainz et al., 2010 and this study), but while *Diporeia* R/D was weakly related to depth, there were no clear, consistent patterns between *Diporeia* R/D, season and dreissenid presence. Similarly, Nalepa et al. (2006a) found that *Diporeia* lipid levels increased even though the population was decreasing at a southern Lake Michigan site. Thus, we suggest that future studies could further evaluate *Diporeia* physiology and nucleic acid responses to environmental conditions since little is known about these phenomena. Moreover, Nalepa et al. (2006a) observed a lack of recruitment by young of year *Diporeia* as they declined from a Lake Michigan site. We were unable to quantify young of year *Diporeia* condition due to the lack of *Diporeia* young of year in our samples. Perhaps, young of year *Diporeia* were absent from our study sites or small enough to pass through a 500 μm sieve and be unnoticed by the naked eye (Johnson, 1988). Nonetheless, we suggest that future research should target early ontogenic stages to hopefully further elucidate mechanisms underlying the decline of this important Great Lakes amphipod.

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