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Reproduction and genetic detection of veligers in changing *Dreissena* populations in the Great Lakes

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Abstract. Dreissenid bivalves, *Dreissena polymorpha* (zebra mussel) and *Dreissena bugensis* (quagga mussel) are biofouling species that invaded the Great Lakes region of North America from source populations in Europe in the 1980s. Initially, *D. polymorpha* spread faster and farther; however, *D. bugensis* have recently displaced *D. polymorpha* in many areas of the Great Lakes and was the first to be found west of the Continental Divide. Early detection of dreissenids is important in anticipating and preventing potentially high economic impacts. To study population dynamics and to enhance detection methods, we assessed "spawnability" using a serotonin bioassay and developed a new, sensitive, multiplex PCR method to identify veligers and verify adult species. Contrasting riverine populations were identified in the Saginaw River (100% *D. polymorpha*) and the Detroit River at Belle Isle (100% *D. bugensis* in 2010), and mixed populations of mussels (10% to ~50% *D. polymorpha*) were found in Saginaw Bay, Lake Huron. In 1994, when the Detroit River population at Belle Isle was virtually all *D. polymorpha* (Ram et al. 1996), spawning could not be induced by serotonin until late May, and peak spawnability did not occur until early June. In 2010, *D. bugensis* at the same site could be induced to spawn in the first week of April, and reached near maximal spawning intensity by mid-May. In 2010, Detroit River veligers were first observed in April and, by PCR species-specific detection, were 100% *D. bugensis*. Veligers changed to a mixed population of both species later in May and rose to a peak, mixed population in early June. These experiments demonstrate a quantitative, species-specific detection of dreissenid veligers, and lay the groundwork for determining the role of early reproduction and other mechanisms in mediating the displacement of one species by a closely related "cousin."

Key words: Detroit River; *Dreissena bugensis*; *Dreissena polymorpha*; Great Lakes; multiplex PCR; quagga mussel; Saginaw Bay; Saginaw River; spawning; species-specific primers; veliger; zebra mussel.

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INTRODUCTION

Dreissena polymorpha (zebra mussel) and *Dreissena bugensis* (quagga mussel) are invasive biofouling bivalves that cause great ecological and economic concern. Detecting them and monitoring their reproduction is important in anticipating and preventing potential high economic costs, and in understanding their ecological impacts. The present study describes populations of these mussels at several sites in the Great Lakes region of North America where the proportions of these two species have recently changed and applies established and

new techniques for monitoring their reproduction.

Native to water bodies of the Ponto-Caspian region of southwestern Asia, Dreissenid mussels have prolifically spread throughout Europe and North America in the last century. Initially *D. polymorpha* expanded rapidly and was the dominant species in the North American Great Lakes, but its presence was limited to nearshore regions (Carlton 2008, Nalepa et al. 2010). However, *D. bugensis* has recently become the dominant species in the Great Lakes and is found in a wider range of habitats than *D. polymorpha* including nearshore and offshore regions (Nalepa et al. 2010). Recently, they were discovered in the Colorado River watershed and other locations west of the Continental Divide in the United States (Benson 2010*a*, Benson 2010*b*, Hickey 2010, Mueting and Gerstenberger 2010).

Dreissenid mussels are considered ecological and economic pests largely due to their prolific reproduction, their capability of adhering to objects, their high capacity of filtering planktonic algae, and displacement of native mussels. Individual females are able to release over 30,000 eggs (Stanczykowska 1977), with some estimates ranging to 1,000,000 or more eggs per female per season (Walz 1978, Borcherding 1991). Release of eggs and sperm in mussels is mediated internally by the neurotransmitter serotonin (Ram et al. 1996), which can be used in the laboratory to assess reproductive capability. Spawning may be synchronized in the environment by temperature and chemical signals from algae and gametes of neighboring animals (Ram et al. 1996, Hardege et al. 1997), resulting in highly efficient external fertilization. High densities of resultant larval mussels (veligers) develop while floating in the plankton for several weeks and subsequently settle and adhere to hard objects, including other mussels and commercial structures such as water intakes of power and water companies.

Settlement of dreissenid mussels on unionid mussels severely reduces the viability of many native mussels (Schloesser et al. 2006, Strayer and Malcom 2007), while other ecological effects may result from the mussels' active filtration of plankton from surrounding waters, leading to a decrease in food resources for planktivorous fish and resultant changes in fish populations (Thorp and Casper 2003). Additional extensive ecological impacts are presented in Ludyanskiy et al. (1993) and reviewed in Ram et al. (2009).

Dense settlement of dreissenid mussels on man-made structures such as water intakes of water and power companies, navigational buoys, and fishnets account for many of the economic costs of dreissenid mussels. Mussels that settle in the pipes of raw water users have ready access to algae and other plankton that also enter the water intake, resulting in few limitations on growth if left undisturbed. Resultant high densities and large clusters of mussels can lead to higher water resistance and even blockage of the plumbing. Early in the *D. polymorpha* infestation of North America, the Monroe, MI Water District reported complete blockage of their water intake by a combination of *D. polymorpha* and frazzle ice (LePage 1993*a*) and the expenditure of more than $300,000 for remediation and engineering to clear out tons of mussels and to devise control measures to reduce future infestations (LePage 1993*b*). The Detroit Edison Monroe Power Plant reported expenditures of about $200,000 during the initial period of their infestation, in 1989–1991 (Kovalak et al. 1993).

While initial costs of dreissenid mussels in North America were high, the development of technical methods for handling them and for anticipating their arrival in new areas have resulted in lower than expected actual costs. In the 1990s, estimates of the economic impact of dreissenid mussels in North America ranged as high as several billion dollars per year (the history and continued use by peer-reviewed papers of these high, mostly gray literature estimates is reviewed by Ram and Palazzolo (2008)). A more recent peer-reviewed economic study indicates that the actual total expenditures of power and water companies for handling mussel infestation problems, including the costs of monitoring, preventative measures prior to infestation, lost production and revenues, etc., was less than $500 million for the entire period from 1989–2004 (Connelly et al. 2007). Facilities further from the epicenter of the invasion benefited from those earlier, high cost experiences and were able to monitor and anticipate the settlement of mussels and adopt less expensive control and prevention measures. Hence, an important component in achieving lower costs

from dreissenid biofouling is an effective monitoring program.

Development of monitoring methods specific for *D. bugensis* and understanding differences in their reproduction and ecology from *D. polymorpha* has taken on new importance recently for several reasons: (1) In the Great Lakes *D. bugensis* have begun to displace *D. polymorpha* in many sites of the original infestation; (2) most of the new mussel infestations west of the Continental Divide in North America have been *D. bugensis*; and (3) in Europe, *D. bugensis* has spread recently into areas previously infested only by *D. polymorpha*, including the Don and Volga river basins in the 1990s (Orlova et al. 2004, Zhulidov et al. 2010) and Dutch waters in 2006 and 2007 (Molloy et al. 2007, Schonenberg and Gittenberger 2008).

The present study focuses on the development and use of methods for monitoring the presence and reproductive capability and activity of Dreissenid mussels. A new species-specific multiplex PCR method for verifying adult species and identifying veligers is described. These methods are applied to describe changing populations of mussels in the Great Lakes basin at sites in the Detroit River, Saginaw River, and Saginaw Bay, which have been the focus of previous studies when the predominant dreissenid species was the *D. polymorpha* (Ram et al. 1996, Vanderploeg et al. 2001).

Materials and Methods

Study sites and animal collection and maintenance methods

Dreissenid mussels were collected along the shores of the Detroit River at Belle Isle (environs of 42.35° N, 82.97° W; a map is illustrated with the *Results*) and at several sites in the Saginaw River (environs of 43.60° N, 83.89° W; see map in the *Results*). At the Detroit River sites dreissenid mussels were collected by removing them from steel sea walls, using a steel scraper with a long extension arm enabling mussels to be scraped from the sea walls at depths as great as 3 m below the surface. At the various sites in Saginaw Bay, a large bay of Lake Huron (environs of 43.8° N, 83.6° W; see map in the *Results*), mussels were hand collected by divers. The Detroit River site at Belle Isle is the identical site from which mussels were collected and assessed for reproductive capability in relation to date of collection and water temperature in a previous study, when the population was exclusively *D. polymorpha* (Ram et al. 1996: Fig. 4).

In the present study, mussels and plankton samples were collected from the Detroit River mostly at weekly or biweekly intervals during spring and summer 2010, also measuring water temperature and collecting plankton samples for observation and identification of veligers. Adult mussels scraped from the sea walls were either preserved in 70% ethanol or, for physiological studies, maintained cool on ice until placed in laboratory aquaria within two hours of collection. In the laboratory, mussels were stored in 5-gallon aquaria with water circulation at 4°C and an 8:16 LD light cycle, without feeding, until tested for reproductive capability and/or dissected to assess gonad sex and maturity. On most collection dates, up to seven plankton samples were collected using a Wisconsin Plankton Net with a mesh of 80 μm attached to a 15.2 m line. The net was thrown from the seawall and upon sinking it was maintained at the maximum depth the line would allow for 60 s and then manually retrieved using a hand-over-hand technique at a rate of 0.5 m/s, resulting in approximately 0.19 m^3 of water sampled. Upon retrieval the sample was deposited into a 15 mL vial with corresponding water. For veliger enumeration, samples were immediately brought back to the laboratory and centrifuged at 6000 rpm for 5 min. Supernatant water was removed using a pipette, down to 5 mL of sample plus water, then 10 mL of 90% ethanol was added as a preservative, and vials were stored at 4°C until veligers were enumerated microscopically or analyzed by PCR (described in *Assessment of Reproduction* and *Multiplex PCR identification of* D. polymorpha *and* D. bugensis, respectively). At the Saginaw Bay and Saginaw River sites, where the main objective was simply to characterize the species composition of the current population of adult mussels, collected mussels were immediately immersed in 70% ethanol for preservation.

In addition to mussels collected from the Detroit River, Saginaw River, and Saginaw Bay sites, preliminary experiments also utilized adult *D. polymorpha* collected from the Huron River in Ann Arbor, MI and Cass Lake in Oakland County, MI, both *D. polymorpha* and *D. bugensis*

retrieved from an anchor in western Lake Erie, and ethanol-preserved *D. bugensis* veligers from Lake Havasu, California.

The species of adult dreissenid mussels were distinguished by several characteristics, as previously described by May and Marsden (1992), Claxton et al. (1997), and others; however, the factor that was most reliable, as confirmed by molecular analyses in the *Results* of this paper, was the sharpness of the angle of the carina, the same criterion suggested originally by May and Marsden (1992). A subset of adult mussels was subjected to molecular analysis to verify species identifications. The species of veligers were assessed by molecular analysis.

Multiplex PCR identification of D. polymorpha *and* D. bugensis

Primers for a multiplex PCR assay to detect and differentiate *D. polymorpha* from *D. bugensis* (Table 1) are based on known sequence differences between *D. polymorpha* and *D. bugensis* in the mitochondrial 16S RNA gene and the mitochondrial cytochrome oxidase I (COI) gene. Access IDs of published sequences of these genes used for design of these primes include the following: AF038997.1, *D. polymorpha* 16S ribosomal RNA gene; AF038996.1, *D. bugensis* 16S ribosomal RNA gene; EU484456, *D. polymorpha* mitochondrial COI gene; and EU484436, *D. bugensis* mitochondrial COI gene. Two 16S primers were designed specifically to amplify a 236 bp DNA amplicon from *D. polymorpha* and nothing from *D. bugensis*; while two COI primers were designed specifically to amplify a 417 bp amplicon from *D. bugensis* and not from *D. polymorpha*. As will be shown in *Results*, these primers produce amplicons for each species that can readily be differentiated by either Tm (melt temperature, using real-time SYBR Green fluorescence PCR) or relative mobility on agarose gel electrophoresis.

DNA extraction.—For assay development and verification of adult mussel species (see *Discussion* regarding ambiguities in species identification) small pieces (~1 mm square) of ethanol-preserved gill or other tissue (e.g., whole animal of about 1 mm length) were homogenized in DNAzol (Molecular Research Center, Inc., Cincinnati, OH), incubated for 15 minutes at room temperature, and then either frozen for later processing, or immediately diluted 50-fold with water, and 1 μL was used as template in PCR.

For PCR of DNA extracted directly from complex plankton samples without isolation of individual veligers, a more rigorous extraction and purification of DNA was necessary. After centrifugation and resuspension in a smaller volume (1.0 ml) of 70% ethanol, plankton samples were transferred to 1.5 mL centrifuge tubes, centrifuged again, supernatant was removed, and the plankton pellet was homogenized in 15 μL DNAzol with a pristine DNA-free disposable polyethylene pestle designed specifically to fit 1.5 mL centrifuge tubes. The homogenate was incubated for 15 min at room temperature. Next, the entire homogenate (~15 μL) was transferred to 275 μL of a DNA extraction medium comprised of Proteinase K solution (20 mg/mL, Invitrogen), 20 μL, plus 255 μL Promega Wizard SV Genomic DNA purification system solutions (catalog no. A2360, Madison, WI: nuclei lysis buffer, 200 μL; 0.5 M EDTA, pH 8, 50 μL; and RNase A, 5 μL) and incubated overnight at 55°C. Finally, DNA was extracted using the Promega Wizard kit's SV minicolumns according to the manufacturer's instructions, yielding a final extracted volume of 500 μL, of which 1 μL was used as template in PCR. Each set of extractions on a particular day was accompanied by a negative control, in which the same procedure, starting with 15 μL DNAzol, was followed for a clean tube that contained no sample. *D. polymorpha* and *D. bugensis* positive controls (Z+ amd Q+) were DNAzol extracts prepared from gill tissue of unambiguously identified adult animals.

PCR and amplicon analysis.—Multiplex PCR reactions consisted of 1 μL template DNA (prepared and diluted as described above, or control solution), 0.5 μL of 20 pmol/μL of each of the primers in Table 1, 12.5 μL iQ SYBR Green Supermix (a 2× pre-mix of *taq* DNA polymerase, Mg^{2+}, buffer, SYBR, nucleotides, etc.; Biorad cat. #1708880 (Hercules, CA), and 9.5 μL water. PCR was performed on a Biorad iCycler as follows: 5 min at 94°C; 35–40 cycles of 30 sec at 94°C, 30 sec at 62°C, and 1 min at 72°C; 5 min at 72°C; a melt curve over the range of 55–95°C; followed by a final hold at 18°C.

Progress of the PCR reactions was monitored and evaluated by a real-time SYBR-Green meth-

Table 1. Sequences of species-specific primers.

Species specificity	Primer name	Primer sequence	Product size
D. polymorpha	ZQ16S147F	AAGACGAGAAGACCCTATCGAA	236 bp
	Z16S383R	AAACTACTGCGCCAAGGAAG	236 bp
D. bugensis	QCOI151F	GATAGGTGGATTTGGAAACTGG	417 bp
	QCOI568R	ACGATCAGTTAAGAGCATTGTTAAG	417 bp

od; however, final conclusions are based on presence, size, and density of bands obtained from running PCR products on 3% agarose gels.

Assessment of reproduction

Reproductive capability of Detroit River mussels was assessed within 5 days of collection by testing responsiveness to serotonin and by microscopic analysis of dissected gonads. Responsiveness to serotonin was typically tested on 15 to 20 animals, with a comparable number of control animals observed using similar procedures without the addition of serotonin. Mussels were individually transferred to 20 mL vials (one animal per vial). After allowing several hours for animals to warm gradually to room temperature, serotonin solution was added to bring the final concentration to 10^{-3} M serotonin. Water was examined at 20 min intervals for sperm or oocytes, and spawning intensity was rated 0 (no spawning) to 4 (most intense spawning), as described previously (Ram et al. 1993). After 4 hours, non-spawning animals were dissected to assess sex and reproductive maturity using squash mounts of their gonads, on a scale of 1 (small undifferentiated cells, impossible to identify sex) to 4 (fully differentiated large oocytes with large germinal vesicles in females and fully motile sperm in males), as described previously (Ram et al. 1993, Ram et al. 1996).

Reproduction of animals in the field was monitored by analysis of veligers in plankton samples. To enumerate veligers, the vial was briefly centrifuged, the supernatant was removed down to 3 mL, the entire pellet was resuspended in the remaining 3 mL, and the entire volume transferred into a 32 mm petri dish, which was then systematically scanned on an inverted microscope with cross-polarized light (Johnson 1995), to count every veliger in the sample. Photographs of a subset of "typical" mussels on each plate were made for subsequent measurements of veliger size. The counted samples were returned to their sample tubes for subsequent DNA extraction and molecular analysis. At least three plankton samples were enumerated per sampling event.

Results

Adult mussel populations in the Detroit River, Saginaw River and Saginaw Bay

The proportions of adult *D. polymorpha* and *D. bugensis* in dreissenid populations at several sites in the Great Lakes and tributary waters in southeast Michigan is illustrated in Fig. 1. At the Belle Isle site in the Detroit River (Fig. 1A), which 15 years earlier (Ram et al. 1996) had been virtually all *D. polymorpha,* all animals evaluated were now *D. bugensis* (100% of more than 500 adult dreissenids assessed). In contrast, 100% of 100 animals assessed from the Saginaw River are *D. polymorpha* (Fig. 1B). Saginaw Bay currently presents a mixed population, with mussels from 5 sites ranging from 10% to 50% *D. polymorpha* (100 animals assessed at sites A, C, D, and E; 37 animals assessed at B). These readily available populations of both species of North American dreissenids provided biological material for developing and testing a multiplex PCR dreissenid mussel identification method.

Multiplex PCR identification of D. polymorpha *and* D. bugensis

Adult mussels whose morphological features marked them distinctively as *D. polymorpha* or *D. bugensis* were used as positive controls in the development of the multiplex PCR assay. Initially, several species-specific sets of primers were designed and tested separately on DNA extracts from gills of individual animals (Fig. 2). While we could amplify PCR products of different sizes from 16S rRNA gene, we obtained the best separations for both size of PCR product and differences in amplicon Tm using primers targeted at different genes in the two species. Fig. 2

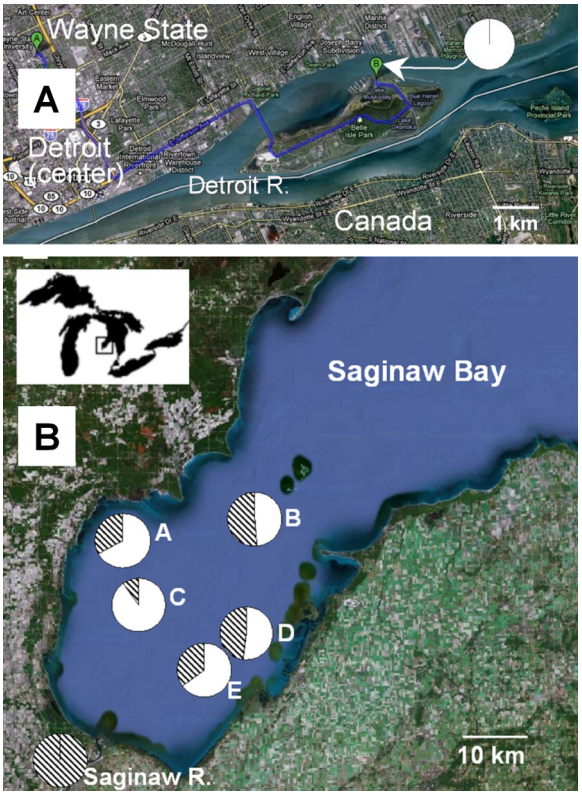

Fig. 1. Proportions of *D. polymorpha* and *D. bugensis* at various collection sites in (A) the Detroit River at Belle Isle, and (B) Saginaw River and Saginaw Bay. Proportions of mussels are represented by hatched (*D. polymorpha*) or clear (*D. bugensis*) sections of pie charts drawn at the approximate position of the collection sites and are based on more than 500 mussels assessed at Belle Isle, on 100 animals assessed at each of the other sites, except for site B in Saginaw Bay, at which 37 animals were assessed due to a smaller number collected.

show the excellent separation of both product size (lane 1 vs. lane 6, Fig. 2A) and amplicon Tm (Fig. 2B) obtained using the primers listed in Table 1.

Combining the 4 primers listed in Table 1 into a single reaction, as described in *Materials and Methods*, we demonstrated that the primers would work just as well in a multiplex reaction. Representative tests of DNAzol extracts from gills of 10 Saginaw Bay animals produced a single amplicon in every case (Fig. 3A). Fig. 3 also illustrates the use of multiplex PCR for verifying species identity of adults, as several of these animals were chosen as "hard to identify" due to variations in shell shape that gave them some of the features that others have suggested differentiated the species. Similar PCR verification tests on more than 100 mussels from the Detroit River, Saginaw Bay, Saginaw River, Huron River, Lake Erie, and Cass Lake indicated that, for morphological identification of the two species, the angle of the carina was the most reliable feature in distinguishing the species. In case of doubt, the multiplex PCR test provides an unambiguous identification.

As predicted from testing the primers separately (Fig. 2) the Tm of the *D. bugensis* multiplex PCR product was higher than for the *D. polymorpha* PCR product, enabling a preliminary judgment of the species to be made from melt curves (Fig. 3B). However, in other experiments (data not shown), other factors in the extracts could shift the Tm, and therefore the conclusion about what species was present was always corroborated by examining the PCR products on agarose gels.

Reproductive capability and activity of dreissenid mussels in the Detroit River

Among several mechanisms that may mediate the change in populations of Dreisenid mussels from *D. polymorpha* to *D. bugensis* may be differences in the reproductive activity between the two species. The site in the Detroit River at Belle Isle that is now all *D. bugensis* is a particularly good site to examine this factor since Ram et al. (1996) previously studied the reproductive capability of mussels from this site when the population was virtually all *D. polymorpha*. We therefore made similar measurements in 2010 as before, of water temperature and "spawnability," as determined by tests of freshly collected mussels with serotonin. These observations were further supplemented with field measurements of veliger densities and identity, using the new molecular tools that we had developed.

Fig. 4 compares the water temperature and serotonin-elicited spawning intensity of mussels collected from the same site at Belle Isle in 1994 (Ram et al. 1996) and 2010. In both years, observations began in April at a time when the water temperature was below 10°C and none of the mussels in either population could be

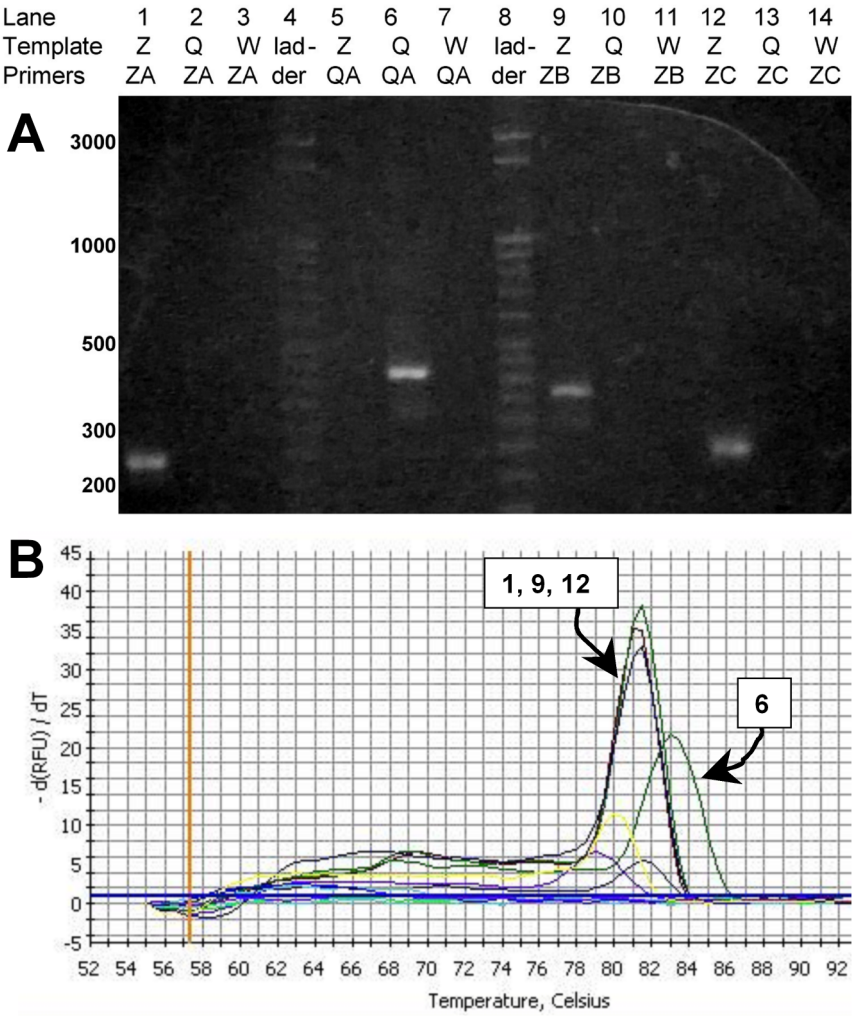

Fig. 2. Test of *D. polymorpha* and *D. bugensis* specific primers. Templates are: Z, DNA extracted from known *D. polymorpha* adult; Q, DNA extracted from known *D. bugensis* adult; W; pure water. Primers are: ZA, ZQ16S147F & Z16S383R; QA, QCOI151F & QCOI568R; ZB & ZC, two other *D. polymorpha* specific mitochondrial 16S primer sets. (A) Amplicons separated on 3% agarose gel. (B) Tm curves obtained for all of the reactions shown in (A). The Tm for the products in lanes 1, 9, and 12 are almost identical (approximately 81°C) despite their range in amplicon size. The Tm for the product in lane 6 (from *D. bugensis*, Tm = 83°C) is greatly shifted from the others.

stimulated to spawn by serotonin. However, in 2010, *D. bugensis* from the site could be stimulated to spawn by serotonin as early as April 10 and April 25, before the water temperature in the field had begun to rise above 10°C. In contrast, in 1994, the *D. polymorpha* from the site could not be stimulated to spawn until near the end of May when the water temperature had risen to around 15°C. The average intensity of serotonin-stimulated spawning exceeded 2.0 in 2010 in mid-May, whereas a similar intensity of spawning was not reached in 1994 until early June. Possibly, some of the differences between the two years could be due to seasonal differences in temperature since the water temperature began to rise slightly earlier in 2010 than in 1994 and ultimately reached a higher temperature, exceeding 25°C in 2010 around June 4, while the highest temperature in 1994 was around 23°C and was reached approximately June 10. With regard to

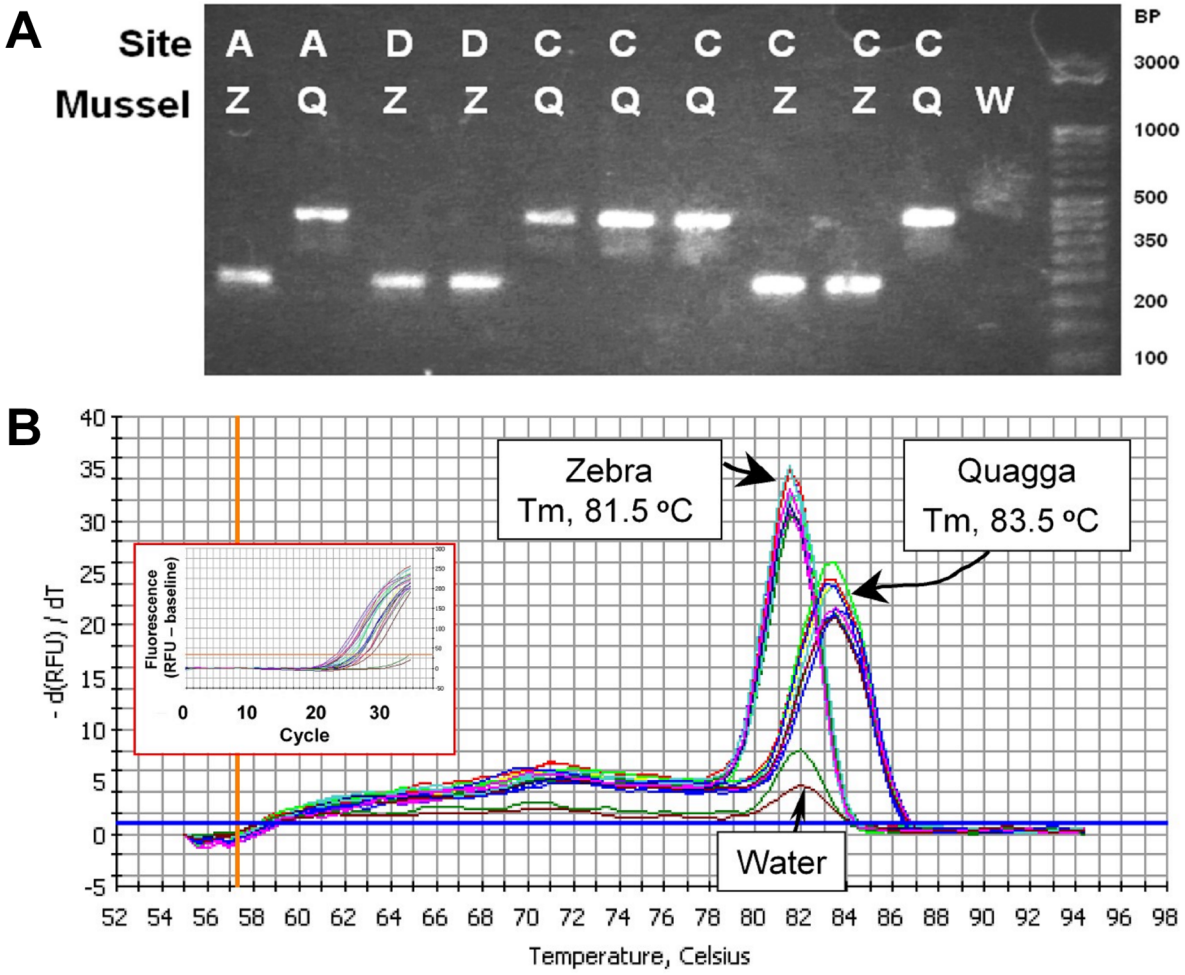

Fig. 3. Multiplex PCR on DNAzol extracted DNA from *D. polymorpha* (Z) and *D. bugensis* (Q) from several Saginaw Bay sites shown in Fig. 3. (A) Electrophoresis of a subset of samples from sites A, C, and D; Z and Q, the suggested species identification prior to the molecular tests (B) real time fluorescence result of the same samples. Positive amplicons were detected between cycle 20 and 25. Inset shows fluorescence during run: Main graph shows melt curves.

the end of the spawning season, several weeks data are missing in July 2010; however, it is evident that when measurements were resumed in August that, as in 1994, spawning intensity has weakened. Nevertheless, some mussels are still capable of spawning in response to serotonin, and this reproductive capacity continued to a much later time in 2010 than in 1994.

In 2010, the above measurements were supplemented by veliger counts in the field near the Belle Isle collecting site (Fig. 5). While a small number of veligers appeared in plankton samples as early as the first week of April, the number of veligers began to rise significantly only at the beginning of May, more-or-less coinciding with the rise in serotonin-stimulated spawning intensity above 2.0. However, the biggest rise in veliger density occurred in a short-lived "pulse" that was observed on June 4, when the average veliger density rose to more than 5 times its previous density. The density of veligers then decreased rapidly over the next few weeks, with veligers continuing to appear in the plankton in lower numbers into the months of August and September.

Veliger measurements quantified changes in

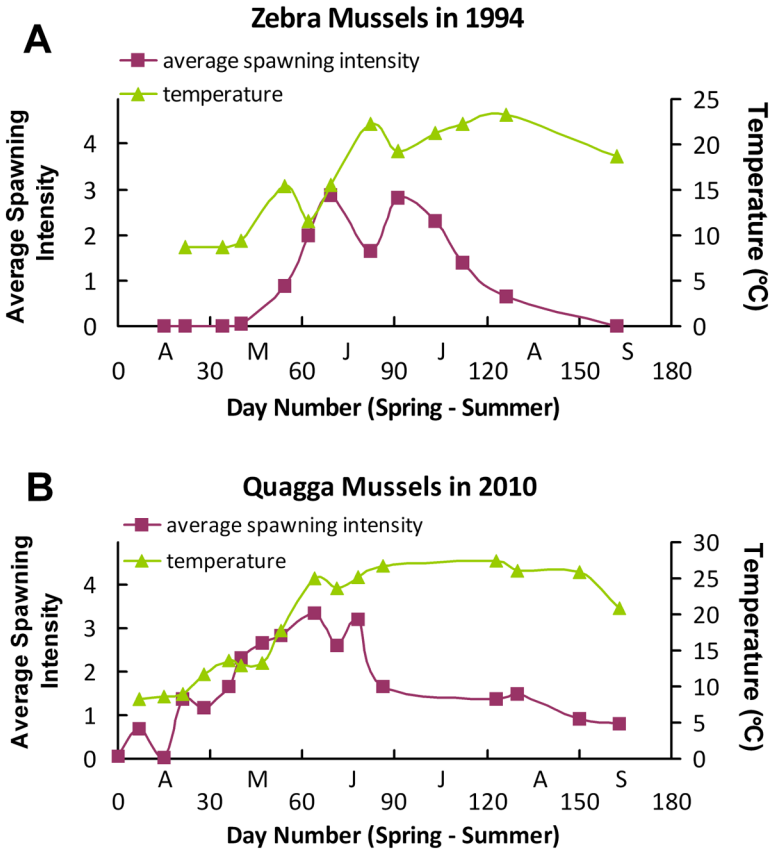

Fig. 4. Spawning in freshly collected mussels in response to 10^{-3} M serotonin. On each indicated date, 40 mussels recently collected from the Detroit River at Belle Isle, Detroit, MI were tested. Water temperature at the collecting sites is indicated. Spawning tests were done at ambient temperature (approximately 22°C). Spawning intensity was rated on a 4 point scale, as described in the text. (A) *D. polymorpha*, collected and tested in 1994 (reproduced from Ram et al. 1996: Fig. 4). (B) *D. bugensis* from the same site collected and tested in 2010.

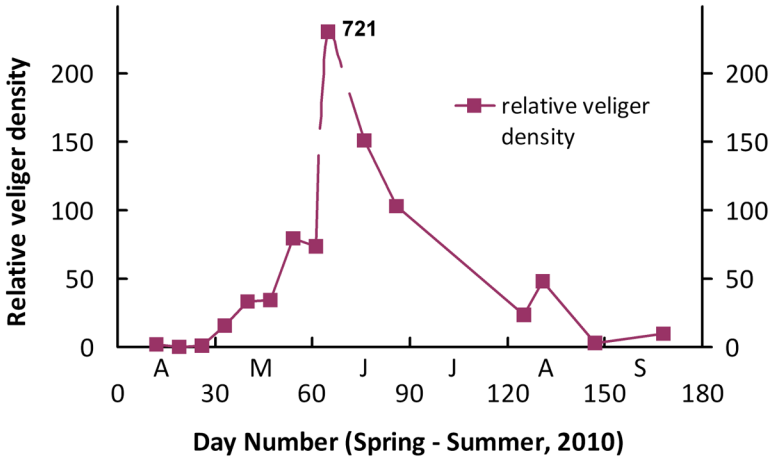

Fig. 5. Veliger densities observed in plankton collected at Belle Isle.

the sizes of veligers as the season progressed. Veligers observed near the beginning of the spawning season were all fairly uniform in size, averaging around 150 μm in length. Larger size veligers (maximum size of 225 μm) began appearing near the end of May and then later, with a big increase, in mid-June. The largest veligers observed in this study were approximately 350 μm in length, observed in mid-June; however, no size measurements were conducted on veligers collected after mid-June.

Finally, we applied multiplex PCR to characterize the species of the veligers. Fig. 6 shows PCR products obtained from plankton pellets analyzed for selected dates during the 2010 spawning season. Early in the season (April 19), no mussels were detected in the sample tested. By May 10, when the veliger density had begun to increase according to veliger counts (Fig. 5), only *D. bugensis* were detected. However, later in May, when larger animals and higher numbers of mussels were seen microscopically, PCR indicated both *D. bugensis* and *D. polymorpha* were present in the plankton, a pattern that continued in June and August. The above observations were repeated with other samples collected on the same dates. A small positive signal was observed in one of three April 19 samples; May 10 showed only *D. bugensis* (4 replicates; one was blank, however); and the other dates always showed bands for both *D. bugensis* and *D. polymorpha*, with June 4 giving the most intense bands. May 17 also had only *D. bugensis* while May 24 had both species (data not shown, one plankton sample each).

Quantitation of PCR results with real-time fluorescence data supports these observations. The 6/4/2010, which had the highest veliger count, consistently exhibited the lowest Ct (the cycle at which the real-time fluorescence rose above background level), indicating the highest dreissenid DNA concentration. Assuming a doubling of amplicon concentration in each PCR cycle we calculated the relative amount of DNA for each sample and obtained an excellent correlation ($r^2 > 0.9$), illustrated in Fig. 6B, between the veliger count and the relative amount of dreissenid mussel DNA in the sample. With its excellent correlation with veliger numbers, PCR Ct measurements with these primers can thus be used to estimate veliger density, with the limitation that a low signal in some negative controls (Fig. 6B) prevents quantitation of low numbers of veligers (<5) in plankton with the described method.

Discussion

Differences in the reproductive capability and timing of spawning of *D. polymorpha* and *D. bugensis* may mediate the on-going transition of dreissenid populations in the Great Lakes from *D. polymorpha* to *D. bugensis*. This study developed a new, quantitative species-specific multiplex PCR assay to verify adult populations and identify planktonic species composition. Together with standard methods for assessing spawning responses and the presence of veligers in the field, these methods were used to characterize the changing populations of dreissenid mussels at several sites in southeast Michigan in the Great Lakes region. The data and methods developed here lay the groundwork for future studies of mechanisms mediating the displacement of *D. polymorpha* by *D. bugensis*.

Although adult dreissenid mussel species can usually be distinguished by a trained biologist, some investigators have reported difficulty. Grigorovich et al. (2008*b*) reported that morphological criteria such as the shape of anterior muscle scars sometimes disagreed with their sequence-based assignments and that the shape of the carina varied greatly within each species, making identification based solely on these morphological criteria problematic. In their original description, May and Marsden (1992) noted that the "normally wide intra-specific variability in shell shape and coloration can lead to uncertain identification." *D. polymorpha* and *D. bugensis* veligers are also difficult, if not impossible, to discriminate by morphological criteria alone (Claxton and Boulding 1998). In some cases, distinguishing morphological criteria can be destroyed by breaks or deformations of their shells occurring during normal development of the mussels (Claxton et al. 1997). Indeed, the temperature at which *D. bugensis* develop has been shown to change shell morphology (Peyer et al. 2010). As a result, the development of molecular methods for distinguishing dreissenid species is important for accurate analysis of both adult and juvenile dreissenid populations.

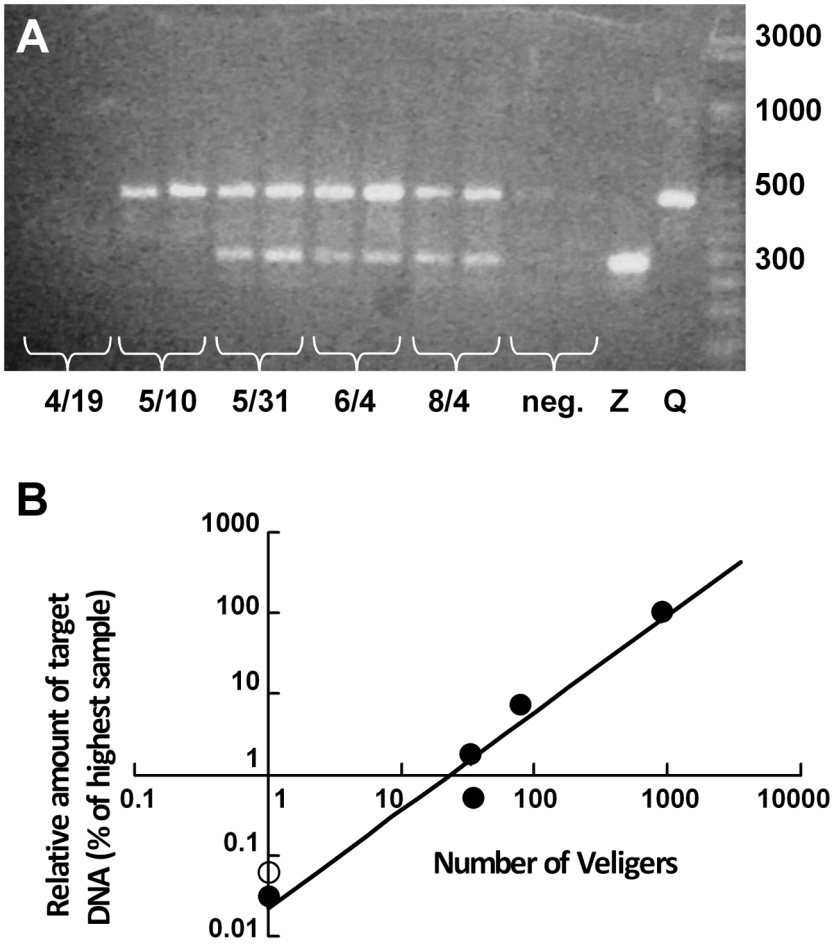

Fig. 6. Multiplex dreissenid PCR of DNA extracted from plankton pellets from various dates. (A) Amplicons separated on 3% agarose gel. Two lanes are shown for each date as each extract was assayed in duplicate PCR reactions. neg., a DNAzol negative control; Z, *D. polymorpha* positive control; Q, *D. bugensis* positive control. (B) Correlation of number of veligers counted in a sample v. relative amount of dreissenid DNA determined from the real time PCR Ct values. Filled circles: results from plankton samples. Open circle: negative control extraction from an empty plankton tube. The amounts of DNA were calculated as per cent of the sample with the largest number of veligers according to the formula $100 \times 2^{-(\text{Ctobserved}-\text{Ctfor6/4})}$, where Ct observed is the average Ct of the duplicates for a particular date and Ctfor6/4 is the average Ct for 6/4/10 (=cycle 19.075). To avoid log(0), the graph used a minimal value of 1 for the 4/19/10 sample and the negative control. The line is a linear regression between log (veliger count) and log (relative amount of DNA), for which $r^2 = 0.952$ (or 0.910 if the 4/19 sample is omitted).

The multiplex PCR assay demonstrated here has advantages over previously reported molecular assays of dreissenid mussel species. The original differentiation of the species in North America by May and Marsden (May and Marsden 1992) described species-specific allozymes that could reliably identify the species; however, its application to veligers has not been demonstrated. Sequences of mitochodrial DNA sequences have been used by many investigators to distinguish the species, based on sequences of COI (Baldwin et al. 1996, Grigorovich et al. 2008*b*, Quaglia et al. 2008, Schonenberg and Gittenberger 2008), cytochrome b (Stepien et al. 2005), or 16S rRNA gene (Stepien et al. 1999, Grigorovich et al. 2008*a*). Using these sequences, several investigators developed PCR-based methods in which conserved primers were used

to amplify mitochondrial genes in both species, followed by restriction enzyme digests to identify species-specific restriction fragment patterns (for COI, (Baldwin et al. 1996, Claxton et al. 1997, Claxton and Boulding 1998); for 16S rRNA gene, (Stepien et al. 1999)). While these methods can detect the presence of mussels, including veligers, identification of the species requires post-PCR processing, such as sequencing or analysis of restriction digests. A set of species-specific PCR primers based on 28S rDNA gene sequences has recently been described (Hoy et al. 2010). The single-tube multiplex PCR assay developed here is much simpler.

Prior to developing the primers described here, we designed *D. bugensis* - specific primers for the same sites on mitochondrial 16S rRNA gene as the *D. polymorpha* primers in the present study (Ram et al. 2009). While this assay (as well as the recent primer set by Hoy et al. 2010) successfully differentiated one species from another without the need for sequencing, the products for both species are the same size, requiring each sample to be tested in multiple tubes, one for each primer set. Using primers that produce different-sized products assists in quality control as the product size immediately identifies not only what DNA is present but also what primers have been used. Different-sized amplicons also have the advantage that they can often be differentially identified in real-time mode by their Tm differences, although we caution against drawing conclusions regarding species without confirmation on agarose gels. The species-specific primers designed here can be used for multiplex PCR, thereby reducing the numbers of tubes needed for the assay and, as demonstrated in this paper, enabling the detection, identification, and quantitation of dreissenids in plankton samples in a single tube assay. Experiments to enhance the sensitivity of the assay to low numbers of veligers and to identify individual veligers are in progress.

After describing several populations of mussels in southeast Michigan, this molecular species identification technology was used, along with standard methods, to characterize reproductive activity and to compare it to a previous study in the Detroit River when *D. polymorpha* had been predominant. The data show that *D. bugensis* are precocious in their reproductive development compared to *D. polymorpha*. Three aspects of the data support this: First, compared to the previous study (Ram et al. 1996) at the same site, *D. bugensis* became responsive to serotonin-elicited spawning activation several weeks earlier in the spring. Second, veligers began appearing in the water coincident with the increasing responsiveness of *D. bugensis* to serotonin. Third, molecular characterization of the veligers showed that while only *D. bugensis* veligers were present in the plankton for the first few weeks that spawning in the field was occurring, *D. polymorpha* veligers later appeared in the plankton samples. Very likely, the smaller veligers initially observed in the plankton were from local *D. bugensis* populations, including the mussels at Belle Isle. *D. polymorpha* began to spawn weeks later, contributing to the total set of veligers, but, according to the relative densities of their amplicons on agarose gels and the very small shift in Tm when they were present, never constituting a larger proportion of the veliger population than *D. bugensis*. Possibly, the largest veligers may be from distant *D. polymorpha* populations; however, molecular assays of individual veligers of different sizes would be necessary to determine if that were the case, and this was not done in the present study.

These data lay the groundwork for further investigations of the mechanisms mediating the change in dreissenid populations from *D. polymorpha* to *D. bugensis*. Despite their superficial resemblance, numerous differences between the two species have previously been described. An analysis of respiration and growth determined that *D. bugensis* have lower respiration but grow to greater lengths and weight than *D. polymorpha*, which may indicate that *D. bugensis* reserve more energy for reproduction and growth (Stoeckmann 2003). *D. bugensis* have greater energy reserves in their larger body size, which may assist them to withstand stressful conditions, such as mid-summer heat, and thereby displace *D. polymorpha* in well-established areas, especially if global warming elevates peak summer-time temperatures at those locations. Other reported differences between *D. bugensis* and *D. polymorpha* include faster and more variable byssal thread production in *D. polymorpha* than in *D. bugensis* (Peyer et al. 2009), a more variable shell morphology in *D. bugensis* than *D. polymorpha*

(Peyer et al. 2010), a relatively larger field of papillae around the siphons in *D. polymorpha* than in *D. bugensis* (Smith 1999), and higher thiaminase activity in *D. bugensis* than in *D. polymorpha* (Tillitt et al. 2009). The higher thiaminase activity of *D. bugensis* may indicate that *D. bugensis* will have bigger impacts on fisheries than previously thought (Tillitt et al. 2009).

Differences in reproduction, such as those we've observed, may also be important. Although both species can readily be stimulated to spawn with serotonin (Miller et al. 1994), Stoeckmann (2003) observed that *D. polymorpha* spawn more eggs than comparably sized *D. bugensis*. The greater fecundity of *D. polymorpha* may provide an explanation for the more rapid dispersion of *D. polymorpha* in the early stages of the dreissenid invasion. *D. bugensis* seem capable of growing at greater depths than *D. polymorpha*, a characteristic that may explain the availability of *D. bugensis* recruits even when *D. polymorpha* are the predominant species at shallower depths (Nalepa et al. 2009). The present study agrees with previous reports that *D. bugensis* become reproductively mature earlier in the season (Stoeckmann 2003) and spawn at lower temperatures than *D. polymorpha* (Roe and MacIsaac 1997, Claxton and Mackie 1998). Similar to the present study, Stoeckmann (2003) observed that *D. bugensis* at 9°C could be induced to spawn by serotonin; whereas, *D. polymorpha* could not. In a recent study describing changes in dreissenid populations in southern Lake Michigan Nalepa et al. (2010) identified differences between adult *D. bugensis* and *D. polymorpha*, yet in describing reproductive patterns the resolution of the veliger data did not allow for differentiation between species. For the Nalepa et al. (2010) study this was not a great concern because at the time of the most recent sampling no adult *D. polymorpha* were found suggesting that the veligers were all *D. bugensis*. However, for early detection of species a technique is needed to identify species specific veligers.

The present study is the first of which we are aware that characterized seasonal changes in the veliger population with an affirmative molecular test to verify that *D. bugensis* veligers are the first to appear in the plankton.

Although *D. bugensis* mussels begin spawning earlier in the season, the highest density of veligers in the present study occurred in a short lasting peak that contained veligers from both species (Fig. 6), raising the question of whether quantity or priority of spawning is more important in establishing eventual predominance. Brief seasonal peaks in veliger densities have previously been described, leading to speculation about factors that synchronize dreissenid spawning, including temperature, pheromones, and phytoplankton (reviewed by Ram et al. 1996). If chemical factors from pheromones and phytoplankton play a role in triggering spawning, then the responsive species may have advantages mediated by these information cues.

The populations of mussels identified here in southeast Michigan provide a source of biological material and environments that may be helpful in studies of the replacement of *D. polymorpha* by *D. bugensis*. The Detroit River and the Saginaw River are two nearby riverine environments with vastly different populations of mussels that may enable comparative studies. Saginaw Bay, with its mixed population, may enable the study of populations in a shallow bay in transition. A survey of other Detroit River sites recently identified populations that are up to one-third *D. polymorpha* (P. Acharya and D.R. Kashian, *personal observation*). Future studies in the Detroit River could determine whether these sites are still in transition to *D. bugensis* or if these relict populations of *D. polymorpha* are sustained by recruitment of *D. polymorpha* or other special conditions of the sites. These studies may lead to a better understanding of factors that enable one species to displace another, and thus a better understanding of biological invasions in general.

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