

Evaluation of Lower Green Bay benthic fauna with emphasis on re-ecesis of *Hexagenia* mayfly nymphs

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Abstract

The last historic *Hexagenia* specimen in lower Green Bay was officially recorded in 1955. Field surveys and *Hexagenia* viability studies were completed to determine if lower Green Bay could support *Hexagenia* re-ecesis and where in the bay egg stocking could best be accomplished. The invertebrate field data were compared with historical population data based on earlier published studies in the 1950s, 1970s and 1990s to determine the bay's ecological trajectory to better understand the re-ecesis success potential of *Hexagenia*. No native *Hexagenia* were observed during this study. Deep water invertebrate diversity within the upper lower bay appears to be improving, whereas the diversity along the lower mid-bay may be deteriorating. Shallower, nearshore samples indicated a better condition with *Caenis* mayflies sparsely present, amphipods, isopods, gilled snails, odonates, oligochaetes, chironomids, and meiofauna present. These results suggested improved conditions shoreward versus degraded conditions deeper. *Hexagenia* egg viability and neonate growth indicated *Hexagenia* could successfully inhabit in situ Green Bay nearshore (< 2 m) substrates; however, deep substrates were generally inhospitable probably due to hypoxia and unstable fluid substrates. As an outcome of the field surveys and studies of *Hexagenia* viability in Green Bay mud, *Hexagenia* stocking began in 2014 with the first adults since 1955 emerging in 2016 at several lower bay nearshore locations. Improved water quality from remediation efforts in the watershed could facilitate the return of *Hexagenia* to deeper water.

Keywords

Green Bay; *Hexagenia*; benthos; sediment; diversity

Introduction

Throughout most of the past seventy-plus years, the bay of Green Bay, Lake Michigan, has experienced a chronic degradation in its water quality. Much of this degradation has been due to point and non-point land-use activity-derived sources discharged to the Fox River which feeds into the bay from the south and travels north primarily along the eastern coast of the bay (western Door County). Historically, the Fox River has been an effluent recipient for paper mills, various manufacturing companies, and intensive agricultural use. For example, when paper companies first started manufacturing specialized papers in 1954, PCBs used in their production were discarded into the river unregulated until 1971, when the use of PCBs was ended (Wisconsin Department of Natural Resources 2012, <http://dnr.wi.gov/topic/greatlakes/documents/RAP-UpdateLGBFR2012final.pdf>). In 1972, the U.S. and Canada implemented the Great Lakes Water Quality Agreement, and major efforts to clean up the lower Fox River and Green Bay were begun when the area was designated an “Area of Concern” (AOC).

The ecological degradation of Green Bay has framed the circumstances leading to vast changes in invertebrate populations, notably, the local extinction of the mayfly *Hexagenia* spp., (historically presuming *H. limbata*, *H. bilineata*, and *H. rigida*). The zoobenthos community provides a lens for temporally comparative analyses to understand the ecological trajectory of the bay that is important for determining if *Hexagenia* re-establishment through egg stocking might be timely. A burrowing benthic Ephemeroptera, *Hexagenia* spp. have long been known as an important indicator taxon for their intolerance of poor water quality (Fremling 1989). Prior to 1939, *Hexagenia limbata*, once regionally coined the “Green Bay Fly”, emerged synchronously in large masses on an annual basis (Schuette 1928, Fremling 1968). Since then, the population of the insect dropped to the point where the last *Hexagenia* was officially recorded in the southern bay in 1955 (Balch et al. 1956). Long-term remediation actions

can prove successful in improving the quality of the environment to the benefit of *Hexagenia* re-ecesis. In western Lake Erie, the same mayfly similarly disappeared years ago due to poor water quality and hypoxia. Then, in the 90's, populations of *Hexagenia* returned to the lake after a 40-year disappearance (Bridgeman et al. 2006). This remarkable recovery was attributed to both remedial efforts and the invasion of zebra mussels (*Dreissena sp.*), which initially helped clear the water and reduce the effects of pelagic sedimentation. In 1991, several living *Hexagenia bilineata* (not *H. limbata*) were collected from the lower Fox River tributary to Green Bay (Cochran 1992), an optimistic sign of natural recovery and/or effective remedial efforts in the Green Bay area.

Monitoring population dynamics of a stressed ecosystem over time can link the past, present, and future health of an environment. Because contaminants can accumulate and persist in the bottom sediment of lakes and rivers long after the water has been cleared of pollutants, population changes of benthic fauna are important for understanding the health of the habitat. It is suggested that macrobenthic invertebrates and meiofauna within Green Bay could be responding positively to remedial efforts, and possibly the dreissenid impact. This study describes the current population trends of benthic fauna juxtaposed with historical population studies to better understand the environmental state and ecological trajectory of Green Bay as referenced to re-establishment of *Hexagenia*. A comparison was made between our data (this study), Surber and Cooley (1952), Howmiller era data 1969-1977 (Mozley and Howmiller 1977, Howmiller and Beeton 1971), and Harris' survey data in 1978 (1998) to determine the directional shift of the benthos. Howmiller and Maas (1973) sampled southern Green Bay for benthic fauna in 1969 and in 1970, leading Howmiller and Beeton (1970) to make predictions about future changes in population dynamics assuming the continuation of the incidence of eutrophication and pollution at that time. Postulations were that populations of oligochaete worms would decrease near the mouth of the Fox River and in the southernmost area of the bay in general, and midge larva populations

would decrease in the northern stations (Mozley and Howmiller 1977). These changes may not have been the general association of these worms to degraded habitat (increased worms, e.g., *Limnodrilus* spp. signifying a degraded habitat), but rather related to a complex hypoxia situation (Klump et al. this issue) and inhospitable sediment changes resulting in decreased worms and severely degraded habitat. These relationships are closely tied to the ecological events that decimated the historical native *Hexagenia* population and to the future possibility for a restored *Hexagenia* population.

The consolidation of sediment became a topic of interest when sediment collected from some Green Bay sites with known historical *Hexagenia* populations appeared to have little cohesive structure. For burrowing nymphs of *Hexagenia*, highly fluidized substrate with little structure leads to the collapse of burrows making the substrate bioenergetically uninhabitable (Fremling, C. personal communication to Kaster, J.L., 1968). Sediment spike tests (Fremling, C. personal communication to Kaster, J.L., 1968) were conducted to rate the cohesive firmness of the sediment that could be crucial to the survival of obligate tube-forming organisms, including *Hexagenia* and certain tubificid oligochaetes. From 2011 to 2015 laboratory rearing of *Hexagenia* from eggs and nymphs was used to provide a window into the effects of fluidized sediment on burrow construction and general background pollution on the early life-stages of *Hexagenia* (Groff and Kaster 2017). This suite of field and *Hexagenia* viability lab studies indicated that nymphs successfully hatched as adults in sediment collected from the southernmost portion of Green Bay, and it followed that in-situ field conditions may be able to support *Hexagenia* populations considering both field invertebrate studies and viability studies. Our overriding question was, is it time to facilitate re-establishment of *Hexagenia* in Green Bay through a stocking program? Armed with information from these studies, an aggressive 2014 *Hexagenia* stocking program was initiated in Green Bay. This study examines time-comparative field analyses of zoobenthos population ecology, enclosure *Hexagenia* viability studies, and *Hexagenia* stocking program in lower Green Bay.

Methods

The target *Hexagenia* stocking sites within the lower bay were: Sawyer Bay 421ac (part of Sturgeon Bay); Little Sturgeon Bay 395ac; Little Tail Point 1334ac; and Long Tail Point 1248ac, and Fox River De Pere impoundment 600ac. The area of these sites total 1629 ha (3998 acres), an area that did not include nearby areas that had historical high abundances located in the middle of the AOC. Considering the mass swarming and dispersal behavior of adults, the project's area being stocked could serve as an inoculum source as the lower bay impact is mitigated.

Field Studies

Macrobenthos. The bulk of benthic field samples were collected on June 21-June 23, 2011, in Green Bay aboard the R/V Neeskay and additional summer samples primarily collected smaller craft from 2012-2016. In total 34 sampling stations were chosen from a grid-pattern arrangement of sites throughout the lower bay (Fig. 1). The open lake sites sampled included 0, 2 (Hx15), 3, 3A, 5, 6, 8, 9, 10, 12, 17, 18, 20, 21, 22, 24, 25, 26, 27, 42, 43, 44, 47, and H7, H7A, H7B and H8 in the nearshore south bay, S1, S2, S3 (H12), H12A, Hx11 in Sturgeon Bay, and HR9 and HR10 in the Oconto and Menominee River estuaries, respectively (Table 1). Emphasis on sampling the lower portion of the bay ensured that as many sampled sites as possible were in the proximity of historical sampling sites of Surber and Cooley (1952), Mozley and Howmiller (1977), and Harris (1998). Three standard sized Ponar grabs (~23×23 cm) were collected at each station, and three spike cohesive test replicates were taken for each location. At the first sampling sites, when a lack of cohesiveness was first observed, only one trial was completed, e.g., 0/1 or 1/1). Sediment debris and fauna samples were sieved through a 0.5 mm screen and the remaining detritus and animals from each grab were stored separately and preserved in 70% ethanol. It is important to note that practices of collection have varied over the years, e.g., Mozley and Howmiller (1977) used an Ekman grab to collect benthos, whereas Harris' 1978 study

(1998) used a Ponar grab. A conversion adjustment between Ekman and Ponar was not applied in this study.

All sorted fauna from each grab sample were placed in a scintillation vial, and counted and identified as they were sorted from debris using dissection microscopes, compound microscopes when necessary, and a multiple-tally counter. Numbers of each taxon from the three Ponar grabs at each site were averaged and expressed on a m⁻² basis. Samples from site 44 were not sorted or documented as they were predominated by fine manganese nodules with the observation of no living macroinvertebrates.

Data were converted into a series of maps using ArcGIS and base-maps obtained from the Wisconsin DNR, created in imitation of those presented in Mozley and Howmiller (1977) (e.g., Surber and Cooley 1952) and Harris' thesis (1998). Size-graduated circle symbols were used at similar taxa resolution to show differences in densities of fauna between sites, consistent with the early Howmiller studies (Mozley and Howmiller 1977). Unfortunately, the original Mozley and Howmiller data are not available (Mozley pers. com. to Kaster 2012). These maps were juxtaposed with historical maps to determine population changes from 1952 through 2011. Historical data sites from Harris' 1978 study were obtained from coordinates given by Harris (1998).

Diversity. Diversity indices were calculated using Shannon diversity (1948) and Simpson diversity (1949) and allied computer programs and methods of Krebs (1999). Diversity values were calculated using only taxa in Harris' thesis (Harris 1998) to make a parallel comparison between Harris' sampling method and data, and the current data. Nematodes were present, but not quantified in Harris' thesis, so these values were omitted completely from the diversity calculation. The diversity index

values of selected historical sites were contrasted with current sites. Statistical inference followed Elliott (1993.)

Fluidized sediment and hypoxia. Percent water content was obtained by weighing samples before and after drying for 72 hours at 60°C, and grain size fractions were calculated using the Bouyoucos hydrometer method (Bouyoucos 1936). Water content and grain size fractions are reported as averages \pm standard deviations (n = 3 for each site). A spike test was done as a proxy for fluidized sediment and as an indication of substrate suitability for *Hexagenia* burrow construction. The spike test for assessing mud consolidation was done on the Ponar sampled sediment immediately after release from the dredge by sticking a 0.8 cm-diameter spike into the mud and slowly rotating it while withdrawing the spike. If the hole did not collapse, the mud was determined to be firm enough to support *Hexagenia* nymph's burrowing lifestyle (Fremling, C. personal communication to Kaster, J. L., 1968).

Meiofauna. Six petite Ponar (~15×15 cm) grab samples were taken at each of five sites in June 2016 to determine the presence of neonate *Hexagenia*: Cat Islands (H7b, 44.552N, 88.021W), Longtail Point (H7, 44.615N 88.007W), Little Tail Point (H8, 44.593N 87.991W), Oconto River estuary, and Menominee River estuary. Cat Islands and Longtail Point sites reside within the boundaries of the Lower Green Bay AOC, while the others are outside the AOC. Sediment-laden overlying water from each grab sample was poured into a slide-lock plastic bag. This supernatant liquid slurry containing the epibenthic meiofauna (e.g., neonate *Hexagenia*, ostracods, copepods) was subsampled in the lab. Prior to inspection, the volume contained in each sample was measured via 1000 mL graduated cylinder. Six 2-mL subsamples were taken from each sample with a Hensen-Stempel pipette after stirring the sample

to suspend fauna. Subsamples were inspected under a 10x dissecting microscope and meiobenthon abundances were recorded. After a given subsample was inspected, its contents were placed back into the original sample container prior to taking the subsequent subsample, ensuring all subsamples contained the same volume ratio to the total volume in the whole sample.

To estimate meiofauna abundance in a grab sample, the number of individuals in each subsample was divided by the ratio of the subsample volume (2 mL) to the total volume of the sample it was taken from. As six subsamples were taken, this process was replicated six times per sample, and thus sample averages ($n=6$) for total abundance were calculated. Averages were calculated for each site sampled based on the averages for each sample taken from the site ($n=6$). Site-based averages were then divided by 0.023 to give density m^{-2} values (reported as averages \pm standard errors). Statistical significance comparisons between ostracod, copepod, and total meiofauna densities at sites within the Lower Green Bay and Fox River AOC versus sites outside the AOC were calculated using the two-tailed Mann-Whitney U-test.

Taxon richness, evenness, Shannon diversity (H' , \ln) (Shannon 1948) and Simpson's index of diversity (1-D) (Simpson 1949) were calculated for all meiofauna collected in each sample ($n = 6$ per site). Sample values were then averaged to obtain richness, diversity, and evenness values for each study site. Richness (R) refers to the number of taxa units present at a given site. Evenness (E) was calculated as $E = H' / \ln R$. The ratio ($H'/1-D$) of Shannon-Wiener diversity to Simpson's index of diversity was calculated as a metric for determining the importance of rare versus common taxa. Two-tailed Mann-Whitney U-tests were used for comparisons of statistical significance for richness, diversity, and evenness. Statistical inference followed Elliott (1993).

Enclosures. *Hexagenia limbata* of both sexes were collected during an en masse emergence at the public pier in Port Clinton, Ohio, on June 10, 2012. Two coolers filled with about 10 cm of Lake Erie's shore water were placed under a lamppost with swarming mayflies. The collection process took about two hours, after which a thick layer of mayflies had accumulated on top of the water in each cooler. The mayflies were stored in the coolers overnight. All mayflies collected were either subimagos or imagos.

Ova and spermatozoa were stripped from the mayflies by multiple squeezing's by hand into the water in the cooler. Fertilized eggs were transported to the UW-Milwaukee's School of Freshwater Sciences (SFS) in jars on ice. In the lab, eggs were put in oxygenated tanks at 8°C to slow their development, then transferred to 22°C room temperature after three days. Eggs typically hatch after 9-14 days at room temperature, although cooler temperatures can prolong this period for up to 120 days (Wiebe 1926, Fremling 1967, Gerlofsma 1999).

After four days, the eggs were quantified by weight. A group of eggs was "wet-dried" by dabbing with a Kimwipe™. The egg mass was then weighed to 6 places on a Mettler MI5 microbalance. These eggs were returned to water and counted twice under a dissecting microscope using a plankton counting wheel. Five samples were weighed and counted, and all measurements were averaged to find a conversion factor from weight to egg quantity. Quantities of eggs gravimetrically adjusted to about 7500 based on 0.861 µg/egg were weighed and placed in glass scintillation vials. The mass of eggs in each vial was recorded and used to calculate the approximate number of eggs. Vials were filled halfway with GF/F filtered Lake Michigan water and caps were placed loosely on top to permit oxygen flow without allowing too much evaporation.

"Condo" Enclosure Construction and Deployment: Sixteen enclosures designated as "condos" were constructed of plastic milk crates (33 x 33 x 28 cm) lined with 1.2 mm nylon mesh screening on all

four sides and fitted with a solid plastic floor and screwed-on/off lid. A set of 8 condo enclosures (1 condo short-term viability; 7 condos long-term viability) were attached on top of a sub-surface buoyed flat, solid concrete slab (about 1 x 0.7 x 0.1 m thick), with four crates on the bottom level and four on top.

The two short-term egg condo enclosures were marked with tape and a large bolt in the center of the lid to help identify them for SCUBA diver retrieval. Each of these two enclosures was loaded with 9 short-term neonate hatching microcosms constructed of shallow plastic 4-ounce jars (9 cm diameter, 4 cm depth) hot-glued to the enclosure crate floor. A 6.5 cm-diameter circle was cut out of the center of the jar lids and covered with 150-micron Nitex™ mesh to retain the eggs and 2 mm of benthos sediment added to each jar.

The remaining 7 long-term enclosures for each station were loaded with in situ standard Ponar grab mud tested for consolidation. The contents of the grab were emptied into a pan and then filled into each of the 14 long-term enclosure crates to a depth of 3-4 cm. Leftover mud from each grab was put into a large bucket, one for each site, for use in laboratory control aquaria. To load the eggs into the enclosures, a shallow depression was formed in the middle of the mud (to help keep the eggs in place during lowering in the water), and two egg-containing scintillation vials were emptied into the depression. To keep the mud in the bottom of the crates from being stirred and washing out on the way down to the bottom, large garbage bags were put around each condo stack and loosely taped to the bottom crate in two places. The tops of the bags were gathered and secured to a rope with a tie strap, and slits about 10 cm long were cut to allow air to bubble out upon submersion. The four ropes, one from each unit, were attached to a line which was let out along with the enclosure. Once the anchors reached the bottom, the rope was pulled up to remove and recover the bags from the enclosures.

One set of 8 enclosures was deployed at a north site (NC) and a second set deployed at a south site (SC) on June 26th, 2012 off the deck of the R/V Neeskay (Fig 1, Table 1). The two short-term enclosures from each site were SCUBA diver retrieved on July 24th, 2012 off the R/V Neeskay. The long-term enclosures were retrieved on June 25, 2013. Enclosures were placed in an on-board environmental cooler at 10°C, kept on ice for transportation from Sturgeon Bay to Milwaukee, and then stored in an SFS environmental cooler at 7.5°C.

In the laboratory, condo short-term egg viability was examined for neonate hatching by decanting the microcosm water into a first Petri dish, and the next two dishes were loaded with 15 mL of condo mud. Each dish was observed under a dissecting microscope to identify and count *Hexagenia* nymphs present. Once it became clear that the *Hexagenia* eggs had not hatched, the focus shifted from finding neonates to recovering and examining the eggs. Groups of eggs from each jar were identified under a dissecting microscope, removed from the sediment with a BioQuip™ featherweight forceps, and placed on glass slides to be viewed under a compound light microscope. The number of 1) empty (hatched), 2) dormant/viable, and 3) unviable eggs on each slide were recorded. All counted eggs from each jar were placed in a separate scintillation vial with two treatments, water only from Green Bay or Green Bay water along with a small amount (0.1 mL) of sediment from the original hatching microcosm jar. These vials were held at room temperature and observed for hatchlings. When it became clear that many eggs were hatching, the contents of each vial were periodically transferred to a Petri dish to count hatchlings. Neonate nymphs were counted and removed daily for six days. Additional eggs were return to their microcosm enclosures using 4-ounce jars (as above) and re-deployed in Green Bay at Littletail Point, Longtail Point and Cat Islands.

Aquaria Hexagenia Viability: Three Green Bay sediment Ponar grabs were collected at field sites 8 and 47 during the June 21-23, 2011 expedition. These grabs were combined and mixed for each site.

Mud was also collected from the Mississippi River on July 20, 2011 at the boat landing on the Wisconsin side across from Lansing, IA, USA using a petite Ponar. The Ponar was manually pushed into the sediment, then transferred to a cooler. All sediment was transported back to the SFS where it was divided between nine 10-gallon aquaria, three for each site and kept at (17°C). Approximately six centimeters of sediment were placed in each tank, after which the tanks were filled about half-way with dechlorinated Lake Michigan water. The sediment was allowed to settle for 36 hours, after which the tanks were aerated.

Hexagenia bilineata eggs were collected in Lansing, IA from the Mississippi River on July 20, 2011. At about 21:30 hours, truck headlights were shone over the water to attract insects. About 70 subimagos and imagos were collected by hand from the water's surface and from around the headlights. The mayflies were then placed in jars filled with Mississippi River water for transportation. After arrival at the lab, the gametes were stripped and stored in collection jars filled with Mississippi River water in a refrigerator at 7°C until the Mississippi sediment in the aquaria had settled enough to continue the experiment.

Eggs, many of which stuck together in clumps, were weighed using a 6-place microbalance to determine the approximate weight of a single egg so gravimetric estimates could be made in the future regarding numbers of eggs added to experimental petri dishes and aquaria. Before weighing, the clumps of eggs were dabbed lightly on a paper towel followed by Kimwipes™ to remove excess moisture. These eggs were counted using a counting wheel and tabulating counter and an average mass was determined per egg. The weight per egg was recorded by dividing the total original sample weight by the number of eggs counted from each sample. These weights per egg were then averaged from the three samples.

Three more egg samples, measuring around 100 mg, were sorted into separate petri dishes. These were left undisturbed in the lab at 22°C until hatched. The number of eggs was determined by

dividing the original weight of the eggs by the averaged weight per egg from the previous samples.

Viability was measured by dividing the number of hatched eggs by the number of total eggs in each petri dish.

Aquaria were labeled 1-9 (1, 4, and 7 identifying Green Bay, station 8 sediment; 2, 5, and 8 identifying Green Bay, station 47 sediment; and 3, 6, and 9 identifying Mississippi River mud). Around 300 mg of eggs would have been planted in each tank. However, due to a shortage of eggs, aquaria 7, 8, and 9 received approximately 100 mg of eggs. After the eggs hatched, neonate nymphs were collected and counted to determine viability in each of the different sediments.

A 48-hour neonate survival experiment began on August 9, 2011, when *Hexagenia bilineata* eggs were first observed to hatch. Twenty to thirty nymphs were transferred to nine petri dishes containing sediment and water from each of the nine aquaria. Three petri dishes containing mostly water and a small amount of sediment from each site (the Mississippi River, Green Bay station 47, and Green Bay station 8) were also set up in case the nymphs in the previously mentioned dishes could not be observed (since nymphs immediately tunnel into superficial sediment upon hatching). Three additional dishes were filled with only water from each of the three sites along with the nymphs. Survival rates were recorded in each of the petri dishes after a period of 48 hours.

***Hexagenia* Stocking.**

Hexagenia eggs were collected from the Port Clinton, Ohio vicinity (dominated by *H. limbata*) for stocking in the bay of Green Bay; and collected from the Mississippi River in the area between La Crosse, Wisconsin and Fountain City, Wisconsin (dominated by *H. bilineata*) for stocking in the Fox River leading into the bay. Eggs were collected from subimago and imago *Hexagenia* during 2014, 2015, 2016, and 2017 in the Port Clinton, Ohio area during early morning hours. Locations of collection

included the Port Clinton pier extending out past the mouth of the Portage River into Lake Erie, as well as on the walls and windows of well-lit businesses adjacent to the lakefront; streetlights along the pier and bright restaurant signs had attracted the insects to these locations. Several 50-gallon-sized plastic bags full of living or very recently expired mayflies were obtained. To isolate large quantities of eggs and spermatozoa, handfuls were squeezed directly or gallon slide-lock plastic bags of mayflies with some water were squeezed, or mayflies were macerated through a commercial kitchen mesh strainer (the latter method proved better for extracting large numbers of eggs). The eggs were put in a bucket containing Lake Erie water. Eggs sink quickly and accumulated on the bottom, and larger floating mayfly parts were discarded. The eggs were then sealed in slide-lock plastic bags and transported on ice back to the lab. The eggs and any remaining debris were then filtered through a 1.6 mm mesh sieve to remove remaining particulate debris, and decanted yielding a solution consisting almost entirely of only water and eggs. The eggs were further cleaned by washing slowly in finer mesh filters (250 μm , 125 μm), isolating clumps of eggs, which texturally closely resembled fine sand. Eggs were then combined, washed with water from Lake Michigan, placed in a beaker containing Lake Michigan water, and stored at 4°C to ensure no premature hatching would take place. For deployment into each Green Bay *Hexagenia* egg stocking site (Fig 1), the eggs were poured from gallon slide-lock bags directly into the water where they quickly sank to the bottom.

To estimate quantities of the large number of eggs, a predetermined average wet weight per egg (0.861 μg) was determined in 2011 (Barbour and Kaster 2011) as described above. Eggs were dabbed lightly with paper towel followed by Kimwipes™ to remove excess moisture. A laboratory/control hatch rate for eggs to be stocked was determined for comparison to hatch rates in of stocked *Hexagenia* eggs and to condo enclosures. This involved placing 10-14 eggs each into 10 scintillation vials, filled

approximately half way with Lake Michigan water. The number of hatches per vial was recorded, and a mean hatch rate calculated over a 31-day period (2014) and a 45-day period (2015).

Hexagenia bilineata nymphs were collected from the Mississippi River backwater site, “Little Lake” near Fountain City, WI in 2014 and from Ohuta Park, Lake City, MN in July 2015. The Mississippi River *Hexagenia* were used for lab experiments and their eggs used to stock the Fox River where the last reported siting was identified by William Hilsenhoff as *H. bilineata* (Cochran 1992). Twelve petite Ponar grabs were taken at Little Lake (44.157N 91.791W) based on the observation of mayfly burrow holes in clearings between dense macrophytes. Each grab was sieved through 1.6 mm nylon mesh; nymphs were counted and transferred to a cooler containing water from the same area and small fragments of window screen for structure (to reduce stress for nymphs). During the 5-hour return to Milwaukee, oxygen was provided to the water in the cooler via electric air pump/stone. Emerging adult *Hexagenia* samples were collected at Ohuta Park (44.451°N, 92.264W) in Lake City, MN, eggs stripped, and transferred back to SFS and processed as above.

Results and Discussion

Field Studies

Macrobenthon. An overall density decrease of oligochaetes within the entire lower bay was observed within the past thirty years (Fig. 2). Of the sites sampled in 2011, the highest count of organisms was 623/m² specimens at site 5, a south site. In 1969, there were no sites with less than the category 101-1000 oligochaetes/m² (Howmiller and Beeton 1970). Harris (1998) reported that the populations had decreased in between 1969 and 1978. The population has continued its decline, with none of the sites sampled in 2011 containing more than 1,000 worms/m². This represented a considerable change when compared to the sites that housed over 10,000 oligochaetes/m² in 1969 (Mozley and Howmiller 1977). It is also interesting to note that the maximum densities of oligochaetes

shifted from the south lower bay from pre-1980 to further northeast in 2011 (Fig. 2), a region that is now considered one of the highest organically impacted areas stemming from the Fox River. Preliminary diatom microfossil trends suggest eutrophication was highest in the 1970's, followed by a decrease in the early 80's that has remained mostly stable, with a possibly increasing impact of eutrophication in the past few years (Hall et al. 2013). This may correspond to the oligochaete population increasing from 1952 to 1969 and then decreasing from 1969 to 2011 (Fig. 2).

Our results supported Howmiller and Beeton's (1970) predictions concerning fluctuations in oligochaete populations. The populations decreased near the mouth of the Fox River and in the southern bay. Station Hx15 (Fig. 1) just east of the shipping lane presented $<1000/m^2$ oligochaete worms in 2011 compared to an average of $>38,000/m^2$ from 1938 to 1978. *Limnodrilus*, primarily *L. Hoffmeisteri* has become the dominant tubificid oligochaete in 2011, corresponding to Howmiller and Beeton's predictions (1970). The densities of oligochaetes also decreased in almost all other parts of the bay during the same comparative timeframe, which suggests the deterioration of the ecological conditions of the bay's deeper mid-basin despite remedial efforts. The relationship of fewer oligochaete in less organic pollution (cf. Fig. 2 inset comment) is contradictory when "excessive" organic pollution depresses oligochaetes considered pollution tolerant (cf. hypoxia and fluidized sediment section).

Chironomid densities in the north lower bay appear to have stayed about the same from 1952 (Surber and Cooley 1952) to 1969 (Howmiller and Beeton 1971), decreased in 1978 (Harris 1998), and then remained about the same in 2011 (Fig. 3). The south lower bay and easternmost portions of the bay appear to have experienced the least amount of change from 1952 - 2011 and continue to house the greatest densities of midge larvae. Howmiller and Beeton's (1971) suppositions about the changes in midge larva populations since 1969 appear to have been accurate. Where much of the mid- to north lower bay frequently had densities of 500-2,000 chironomids/ m^2 (and Howmiller and Beeton 1971), Harris' 1978 survey (1998) and the 2011 survey commonly yielded only 100-500 chironomids/ m^2 in the

same areas. Decreased chironomid populations were especially depressed in the subfamily Tanypodinae, a largely predatory taxon (Fig. 4a,b,c). Densities of this taxon in the south and eastern portion of the lower bay continue to remain higher than other areas. The subfamily Tanypodinae was recorded by Howmiller and Maas (1973) in two separate genera, *Tanytarsus spp.* (Fig. 4a) and *Procladius cf. culiciformis* (Fig. 4b). Densities of Tanypodinae in 2011 have decreased throughout the lower bay, but most extremely in the northern and mid- reaches of the bay (Fig. 4c).

Notable changes within the past thirty years include the decreased populations of Pelecypoda/Sphaeriidae (Fig. 5), and Isopoda. The latter did not appear in any of the 2011 samples, and fingernail clam populations were reduced to less than 500 organisms/m² at all sites, a significant change since Harris' 1978 sampling in which densities of >2,000 organisms/m² could be found at some of the southern sites. Harris' sites 42 and 60 contained 792 and 2,288 clams/m², respectively, whereas 2011 nearby site 10 contained only six. Although a few species of fingernail clams have been shown to possess high tolerance levels for organic pollution, many are adversely affected. Although clam populations can increase with small amounts of organic pollution (presumably by the addition of nutrients to the system), areas with concentrated pollutants have shown to host small to no populations of sphaeriids (Ingram et. al. 1953).

Nematodes were the most abundant aquatic invertebrate (here considered macroinvertebrates as they were retained while 0.5 mm sieving) throughout the deep water lower bay. The primary nematodes were Monhysteroida, Cyatholaimoidea, and facultative anaerobic taxa. Harris (1998) qualitatively indicated nematode presence/absence; however, nematodes have not been adequately quantified in previous studies, and thus comparisons could not be made. Their widespread abundance may be associated with their tolerance of hypoxia/anoxia and fluidized sediment.

No live gastropods were found at the 2011 sample sites although numerous recently vacated shells were present; whereas live specimens were reported by Harris (1998). Leeches (Hirudinea) were found to be absent at 2011 sampling sites, though small densities were present in Harris' study (1998). A single mayfly specimen was encountered in 2011 at site 10, belonging to the genus *Caenis*. *Caenis* were often found qualitatively at Longtail Point and Littletail Point in 2011. This mayfly was not documented in Harris' thesis (1998), but it was documented by Howmiller and Beeton (1971).

Diversity. Ecological stress recovery usually entails the improvement of "rare" taxa, i.e. taxa that previously have not been present or in low richness, and therefore disproportionately increase H' diversity. This occurrence is usually related to a decrease in the common taxa (perhaps more pollution-tolerant organisms), an increase in rare taxa, or the occurrence of both events simultaneously. Diversity indices applied over time intervals provided a general idea of diversity fluctuations and how population dynamics have changed. Within the population fluctuations observed here there appears to be a trend of gradual improvement of the central north lower bay (e.g., station 26) relative to the south lower bay, e.g., station 6) (Fig. 6). Benthic macrofauna richness, diversity and abundance was relatively low – a condition that has persisted since the 1950's (Harris 1998, Qualls et al. 2013 <http://www.seagrant.wisc.edu/Home/Topics/HabitatsandEcosystems/Details.aspx?PostID=1840>). Benthic invertebrate distribution from the lower-bay is a typical and expected ecological eutrophic pattern (Lundbeck 1936). Ponar collections in 2011 from 18 stations indicated a generally reduced species richness highlighted by high abundance and low Shannon diversity index (4316/m², diversity 1.39, 95% CI=1.36-1.42) at the lower-bay sites (in vicinity of periodic hypoxic episodes), and low abundance and high diversity (163/m², diversity 1.93, 95% CI=1.85-1.98) at the north lower bay sites (Fig. 6). This is probably driven by a combination of the hypoxic condition and the carbon deposition contributing to a fluidized substrate, especially at the mid-bay deep basin (station 36).

The dominant taxa, Chironominae (largely *Chironomus*), Tubificidae (largely *Limnodrilus*), and Nematoda (Monhysteroida, Cyatholaimoidea, and facultative anaerobe taxa), were more abundant at sites episodically subjected to hypoxia. Conversely, Tanypodinae were more equitably distributed indicating a balanced proportion of predatory to non-predatory taxa (Fig. 6). This level of equitability was largely responsible for the high Shannon diversity index in the north lower bay stations. The trade-off between an equitable (e.g., station 26, Fig. 6) and non-equitable (e.g., station 6, Fig. 6) richness distribution has implications on abundance levels that serve as forage to other species (note Chironomidae abundances in Fig. 6).

The central south lower bay showed a general deterioration in diversity. For example, where there were once five and seven taxa at Harris' 1978 sites 42 and 60 (1998), respectively, only four taxa were present in 2011 at station 10, situated between the two historical sites. Diversity data from Harris (1998) compared to our 2011 data indicate a decrease at the central stations of the south bay from 1978 to 2011. (Table 2). Stations Harris 15 and Kaster 5 were near shore rather than in the central bay and showed a increasing Shannon diversity while Simpson diversity remained nearly unchanged (Table 2).

The nearshore south stations (e.g., 5) had the least amount of diversity change of common taxa during the past 35 years. Simpson's index for station 5 decreased only slightly from 0.503 to 0.494, but Shannon diversity values increased from 1.044 to 1.217 (Table 2). This stoichiometry suggests that rare taxa have increased disproportionately compared to common taxa from 1978 – 2011 (Table 2). That is, common weighted taxa (Simpson's diversity, 1-D) have remained about the same, but rare weighted taxa (Shannon diversity, H') increased. As it is the rare taxa of a community that support diversity, this is a positive indication that the bay is tracking toward improved conditions.

Although decreases in the abundance of some common fauna has been seen in the bay, there has been no observation of an increase in species richness. Site 10 lacked Hirudinea (leeches), Amphipoda,

and Isopoda which were historically present in Harris' sites 42 and 60. The loss of the two crustacean groups is indicative of a degraded environment in the central basin. Some taxa were omitted from comparative diversity estimates because of lack of historical quantified information. For example, nematodes were recorded as either "present" or "absent" in Harris' thesis (1998) and not meaningfully mentioned by Mozley and Howmiller (1977). This is surprising observation in that nematodes in our 2011 study were the most abundant taxa in deep waters. They were included in the mid-lower bay diversity indices (Fig. 6). Further investigation would be instructive if the nematode, oligochaete and chironomid specimens were identified to species.

A similar tendency occurred in western Lake Erie, which faced many of the same problems that Green Bay faces today, except for oligochaetes in Green Bay as described previously. According to studies completed by the U.S. Geological Survey in 1982 and 1993, densities of chironomids, oligochaetes, and fingernail clams decreased in Lake Erie as water quality improved and the ecosystem recovered (Ciborowski 2009). The decrease in densities of organisms was coupled with an increase in chironomids making up a large portion of the aquatic fauna (Ciborowski 2009). In the mid-1990s, *Hexagenia* were found in western Lake Erie after a forty-year absence, an occurrence that has been linked to the appearance of zebra mussels which tend to shunt eutrophication processes to the benthos by clearing the water and consolidating sediment (Leach 1993).

Fluidized sediment and hypoxia. The ecological interaction of hypoxia and sediment cohesion have a controlling role in shaping the benthic community. Specific hypoxic events that negatively alter the ecological relations of fauna are not well known. Hypoxic magnitude, frequency and longevity are important limnological features controlling the deep water benthic fauna. While relatively little is known about the community impact of hypoxia, hypolimnetic hypoxia has the capability of severely limiting populations. The dissolved oxygen 96-hr LC50 for *H. limbata* was calculated at 1.4 mg/L (CI=1.2 – 1.6,

$p=0.05$) (Nebeker 1972) and 1.8 mg/L (Gaufin et al. 1974); however, based on our observations the early life history stages (neonates) are probably more susceptible to depressed oxygen levels than are nymphs of later stadia. Others have suggested a 90% loss of *Hexagenia* in most of Lake Erie from a single anoxic event (Britt 1955).

In Green Bay dissolved oxygen concentrations in bottom water fall below 2 mg/L frequently, conditions that often persist for days to weeks (Klump et al., this issue; Grunert et al. this issue). The hardiest hypoxia/anoxic tolerant macroinvertebrates are perhaps tubificids and chironomids. Chironomids, e.g., *Chironomus* spp., under anoxia (and presumably worse case hypoxic conditions) can undergo a facultative dormancy for about 60 days and avoid freezing conditions by burrowing deeper into anoxic sediment (Kaster and Jacobi 1978). While tubificids can also tolerate severe hypoxic conditions, they do not survive in non-cohesive fluidized sediment that cannot support a tube, e.g., *Hoffmeisteri* spp. Comparatively, our observations suggest chironomids can survive both fluidized sediment and severe hypoxia. This may explain *Chironomus* being the last macroinvertebrate taxon present in many deep water Green Bay sites, assuming hypoxia events are of shorter duration than dormancy survival periods. Our Green Bay surveys of 2014-2017 suggested that about 25% of deep water (>3 m) stations with fluidized sediment contained only *Chironomus* while tubificids were absent. Dreissenid mussels, while present in Green Bay, also appear to be limited by dissolved oxygen. Nowhere in the approximate benthic “dead zone” are dreissenid mussels found, although mooring lines and hardware above the thermocline typically colonize with young of the year mussels by late summer. Biofouling of bottom water sensors is virtually nonexistent.

The spike tests indicated that much of the bay’s benthos was characterized by gyttja that was highly fluidized and presented an unstable substrate for benthic fauna, except for the nematodes (Table. 1). The supposition is that fluidized sediment of deeper water will not support *H. limbata*, while

nearshore cohesive habitat may. 8% of the deep-water stations had fully cohesive sediment (100%, Table 1) where as 70% of the shallow water stations had fully cohesive sediment. Both tubificids and chironomid populations are depressed in fluidized sediment even though chironomids appear to survive better than tubificids. When high rates of pelagic primary production, or erosional processes, deliver organic matter to the benthos in excess of what can be processed by tubic-forming benthic fauna, the tolerant fauna either dies (as in heavy organic or silt situations) (cf. fig. 7, Mackenthun 1969) or some emigrate to more hospitable areas (e.g., *Hexagenia*, Fremling, C. personal communication to Kaster, J.L., 1982), even when benthic oxygen levels are adequate. This is a characteristic response for even the hardiest oligochaetes (e.g., *Tubifex tubifex*, *Limnodrilus hoffmeisteri*) under extreme organic input (Kaster pers. com).

Sediment from central-lower Green Bay (stations 2-6) averaged $87.6 \pm 4.5\%$ water. Nearshore sites and river estuaries averaged $43.1 \pm 2.5\%$, $40.0 \pm 1.2\%$, $45.8 \pm 22.5\%$, and $36.0 \pm 13.9\%$ for Longtail Point (station H7), Littletail Point (station H8), Oconto River estuary (station HR9), and Menominee River estuary (station HR10), respectively (Fig. 7). Samples from Little Lake, Upper Mississippi (collection site for *H. bilineata* nymphs used in laboratory survival/growth experiment) registered an average of $51.4 \pm 0.98\%$ water (Fig. 7). Sediment particle size was broadly divided between the mid-bay stations (2-6) and nearshore stations (Fig. 7) with the Little Lake sediments grouped with stations 2-6.

The high-water content, small particle size sediment had an average spike test cohesion of 58% compared with the low water content, large particle size sediment cohesion of 94%. We note that sediment cohesion is a complex characteristic that is not always related to water content as we described above. Groff and Kaster (2017) suggested that *H. bilineata* nymphs were tolerant of fluidized sediment (75% of nymphs survived) and thus may be a better candidate for stocking in or near extensive areas of fluidized sediment in Green Bay, assuming adequate hypolimnetic oxygen. Groff and Kaster (2017)

suggested *Hexagenia limbata* is not well suited to live in fluidized sediment, regardless of adequate oxygen levels.

These sediments grouped into three distinct sand-silt-clay categories regarding grain size fractions: 1. The south lower Green Bay sites (stations 2-6) were dominated by silt (average = $86.0 \pm 10.1\%$). Samples from these stations contained at least 80% silt with most in the 90% range (although one sample at one station registered only 54.8% silt). 2. Sediments taken from Longtail (Fig. 1, station H7) and Littletail points (Fig. 1, station H8) (as well as those from the Oconto (Fig. 1, station HR 9) and Menominee River (Fig. 1, station HR10) mouths and including USGS 2012 data) contained a large percentage of sand (average = $81.1 \pm 5.4\%$ for Longtail and Littletail, and $88.0 \pm 4.5\%$ with USGS data included). 3. Samples from Little Lake, Mississippi River showed a more even distribution with a comparatively high percentage of clay (averages = $21.5 \pm 3.4\%$ sand, $33.3 \pm 3.7\%$ silt, and $45.2 \pm 1.6\%$ clay). *H. bilineata* is more suited to a silty-sand substrate composition whereas *H. limbata* is better suited to sandy-silt. This may partially explain *H. bilineata* dominating the mayfly community in large river systems and *H. limbata* dominating in large lake systems. Siersma et al. (2014) found that in Saginaw Bay, Lake Huron, *Hexagenia* spp. may be returning in low numbers (~ 1.5 nymphs/m²) and had a greater preference for higher sand substrates found nearer nearshore.

Meiofauna. The assessment of benthic habitat quality in Lower Green Bay and the Fox River Area of Concern (AOC) currently relies largely on macroinvertebrate assemblages while entirely ignoring the interstitial meiofauna (45 μ m – 1 mm). Although less commonly used in habitat quality assessment, meiobenthos density and diversity have been suggested to respond negatively to organic enrichment (Raffaelli and Mason 1981, Särkkä 1992). We calculated ostracod, copepod, and total meiofauna densities, taxon richness, evenness, Simpson's diversity index, Shannon diversity, and the ratio of Shannon diversity to Simpson diversity ($H'/1-D$) at two southern near-shore sites within the

Lower Green Bay and Fox River AOC and three sites outside the AOC. All parameters were hypothesized to be significantly greater at sites outside the Lower Green Bay and Fox River AOC. However, our results showed that no meiobenthic density was significantly greater ($p > 0.05$) at a site outside the AOC than at a site within the AOC; and conversely, several metrics were greater at sites within the AOC. In the long-term, meiofauna development should reveal a more robust (as measured by diversity and biological indices) invertebrate community than has generally been gathered by standard Ponar sampling and processing in lower Green Bay.

Total meiobenthos density averaged $20,500 \text{ m}^{-2} \pm 2810$ at Cat Islands, $32,800 \pm 3500$ at Longtail Point, $30,900 \pm 7130$ at Littletail Point, $10,300 \pm 2030$ at the Oconto River estuary, and $21,800 \pm 2180$ at the Menominee River estuary. Ostracod density was significantly greater at Longtail Point than at the Oconto River estuary ($p = 0.031$). Copepod density was significantly greater at Cat Islands than at the Menominee River estuary ($p = 0.046$), and was greater at Longtail Point than at the Oconto River estuary ($p = 0.046$) and at the Menominee River estuary ($p = 0.013$). Total meiofauna density was also significantly greater at Cat Islands than at the Oconto River estuary ($p = 0.025$) and greater at Longtail Point than at the Oconto River estuary ($p = 0.005$) and at the Menominee River estuary ($p = 0.031$) (Fig. 8).

Taxon richness, evenness, Shannon diversity, Simpson's index of diversity, and the rare to common taxa stoichiometry of $H'/1-D$ for all meiofauna sites are given in Table 3. Meiofauna richness was significantly greater at Cat Islands than at the Oconto River estuary ($p = 0.016$) and at Longtail Point than at the Oconto River estuary ($p = 0.005$). Simpson's diversity ($1-D$) was significantly greater at Cat Islands than at the Oconto River estuary ($p = 0.007$) and at Longtail Point than at the Oconto River estuary ($p = 0.010$). Shannon diversity was significantly greater at Cat Islands than at the Oconto River estuary ($p = 0.007$) and at Longtail Point than at the Oconto River estuary ($p = 0.005$). There were

no significant differences in evenness between sites. $H'/1-D$ was significantly greater at Longtail Point than at the Oconto River estuary ($p = 0.008$). No value for ostracod, copepod, or total meiofauna population density or for richness, evenness, Shannon diversity, Simpson's index of diversity, or $H'/1-D$ was significantly greater for a site outside the Lower Green Bay and Fox River AOC compared to a site within. Conversely, several of these parameters were greater at sites within the AOC. The rich meiofauna at these nearshore sites suggests a habitat quality that would support the *Hexagenia* life cycle, especially the meiofauna neonate and post-neonate early stages.

In March 2015, 15 elutriate meiofauna samples collected through the ice at Longtail Point (station 7) yielded 6 *Hexagenia* nymphs which were all less than 2 mm in length. These eggs were stocked at the site in July 2014 as part of the *Hexagenia* stocking program. These were found in samples from two of the three ice holes (approximately 15 m apart), indicating a degree of drift, either as eggs or neonates. The elutriate Ponar samples taken at Longtail Point in June 2015 yielded one live *Hexagenia* nymph on the order of 9 mm (from an elutriate sample). This represents an estimated growth rate of 1 mm/month on average during the first year of a two-year life cycle, noting most of the growth would have been elaborated in the summer months. No *Hexagenia* were found in grab samples from Longtail Point taken on the same day.

Enclosures. *Condo enclosures.* The original sediment loaded into the enclosures had very good cohesive structure (67% cohesive); however, upon their retrieval the sediment in the enclosures was fluidized (0% cohesive) due to the enclosures accumulating settling sediment (probably delivered substantially longitudinally as the condos were covered on top). Several rare taxa encountered via standard Ponar grab sampling were present in the condo enclosures, including Amphipoda, Trichoptera, pulmonate snails (*Ammicola*, *Valvata*, *Bithynia*), and ectoprocts (*Fredericella* spp.). It is not clear how these groups gained entry to the enclosures. Two possibilities are they were present in the loaded

ambient sediment or reproductive adults attracted to the enclosures dropped their eggs that entered through the screening. These taxa were not present in Ponar samples collected at the enclosure sites. Post-analysis, the enclosures were carefully inspected for possible entry point compromises but none were found. Amphipods in the enclosures averaged 461/m² whereas chironomids were 105/m² and oligochaetes 357/m². Average Simpson's diversity was 0.636 for condo fauna; a value that was greater than those collected from 1969-2011 in standard Ponar samples that had a maximum Simpson's of 0.5. Shannon diversity averaged 1.15 for condo fauna that was greater than all values from 1969-2011 Ponar sampling datasets by at least 0.3 units. Taxa richness in condos was 4.86, while 1969-2011 Ponar sampling datasets yielded a maximum average of 4.25 (1978-1994). Taxa evenness was 0.73 for condo fauna, which was also higher than that for any standard Ponar sampling dataset (next closest was 0.67 in 1978). Analysis of taxa using the Modified Family Biotic Index (MFBI, Hilsenhoff 1988) yielded a condo fauna average score of 7.06, indicating a higher habitat quality than from any in situ Ponar sampling dataset.

Of the 1,940 eggs examined from 2012 South condo, 4.6% were empty, 85.3% dormant, and 10.3% unviable (Fig. 9). Of 2394 at the North Station, the empty, dormant, unviable percentages were 9.4%, 80.8%, and 9.8%, respectively. A Mann-Whitney U test determined that the numbers from the two stations were not significantly different ($p > 0.05$) (Elliott, 1993). When these eggs were placed in vials with (oxygenated) water and with or without sediment from Green Bay, the nymphs from the dormant eggs began hatching within 48 hours. Of all the eggs from both condo stations an average of 70% hatched within 7 days. However, the Mann-Whitney U test showed a significant difference ($p < 0.05$) in hatching success between vials with and without sediment: 83.5% and 56.8%, respectively.

Mesocosm enclosures. A *Hexagenia* egg hatch rate of 14% was observed over a period of 32 days for the eggs collected from the mesocosms placed in the field in July 2014. The laboratory control

hatch rate was 25% over a 45-day incubation period. Two live neonates were encountered in the mesocosm recovered from Longtail Point. Ponar grab samples taken at egg mesocosm deployment sites during November 2014 yielded no stocked egg nymphs. This may have been due to an issue with the sampling method—benthic samples were taken via petite Ponar grab and sieved through a 1.6-mm mesh sieve, as opposed to the “elutriate” method. The presence of neonates in both the mesocosms and from sediment grab samples provided firm evidence that *Hexagenia* eggs hatched, grew as neonates (<2 mm long) and grew as juvenile nymphs in their natural setting in Longtail Point, part of the lower Green Bay AOC. This suggested that from the planned 2014 egg stocking, adults could possibly hatch during the summer of 2016, assuming a 2-year life cycle.

Hexagenia Stocking.

Hexagenia eggs stocked nearshore in lower Green Bay were capable of hatching and nymphs capable of growth/development through their full life cycle. To date 4 cohorts (2014, 2015, 2016, 2017) totaling 634 million eggs have been stocked (Table 4). *Hexagenia* eggs stocked in 2014, emerged as *Hexagenia* adults at Sites 7, 7A, 7B, 8, 12, 12A, and 15 during the summer of 2016. Especially notable was the presence of adult exuviae in the AOC (Sites 7, 7A, 7B) and a sizable adult hatch in Sawyer Harbor (Sites 12, 12A). These represent the first *Hexagenia* hatches since the 1950s. Their ability to successfully re-colonize broad portions of lower Green Bay was not fully assessed quantitatively in the timeframe this study allowed, but such results could be expected. The ultimate measure of successful stocking will be the emergence of adults in 2016-2018, followed by their sustained populations in 2018 - 2020, and, beyond. The success or failure of stocking depends largely on favorable environmental conditions and autecology characteristics including survival of early life stages from zygote through the first winter of a 2-year life cycle, and the ability for *Hexagenia* to exceed their reproductive threshold to provide the sheer numbers for sustaining the population. The *Hexagenia* recovery effects of remedial

activities are lagging as proposed by Kolar et al. (1997). Kolar et al. (1997) suggested a 48 – 81-year lag time for *Hexagenia* to reach a level of ~350 nymphs/m² in Lake Erie. This estimate is probably considerably lower when eggs are stocked in mass as in this study. Never-the-less, lag times will place the final success or failure of the stocking effort in the future.

Stocking Sites, Successes and Failures.

Station H7, Longtail Point, H7A Longtail Point, H7B Cat Island. Several hundred adult *Hexagenia* exuviae were observed adjacent to the station by kayak surveillance, with actual counts at 23 exuviae/100 meters on June 23, 2016 within the inner Longtail Point area (station H7). These exuviae were derived from the June 24, 2014 disposition of approximately 28,500,000 eggs. No adults were observed, however 12 subimago exuviae were noted on the vegetation at Station H7. This is evidence that *Hexagenia* can complete their life cycle in the Area of Concern (AOC).

Station H7A is a new station established in July 5, 2016, with the stocking of ~100,000,000 eggs south of the Shipyard Marine marina (44.614N 88.011W). Earlier reconnaissance found a bottom substrate that appeared suitable for *Hexagenia* hatches. August 3, 2016 Ponar elutriate samples were collected and examined in the lab for neonates; however, only 12 individuals were found. Newly stocked *Hexagenia* eggs can take >30 days to hatch, thus few neonate individuals were expected. It is important, however, to note that egg hatching was possible at this AOC site. An adult hatch from this stocking is expected in 2018.

Station 7B, located nearshore between Duck Creek and the Fox River mouth, was established in 2015 with an egg stocking of 40,000,000. The 2017 July hatch was small with only 3 subimago exuviae found on adjacent vegetation. Three *Hexagenia* subimago exuviae were collected from shoreline vegetation. These may have originated from station H7. An additional 100,000,000 eggs were stocked at this site in 2017.

Station H8, Littletail Point. *Hexagenia* stocking and collections. Approximately 29,000,000 eggs were stocked on June 24, 2014 and another approximately 25,400,000 eggs stocked on June 29, 2015. Nymphal exuviae were collected at this station in 2016, June (2/100 m), July (14/100 m), and August (4/100 m). Several hundred (roughly a similar number to Longtail Point) were observed adjacent to the station by kayak surveillance. This group is expected to be derived from the 2014 stocking as the stocking/emergence life cycle indicates a 2-year period (cf. station H12, H12A below).

Stations H12, H12A, Sawyer Harbor. Sawyer Harbor June 21-23 hatch. Exuviae counts along west shore of Potawatomi Island averaged ~95/100 m of shoreline (station H12A). There were many more on the open water surface and winged imagos, but virtually impossible to quantify properly because of their erratic movement. It will be of interest to see if the 2nd naturally reproduced cohort (expected 2018) is successful. It could be years to know if the population will ultimately catch hold. A long-time seasonal resident (on Cabot Point side, station H12) from Des Moines, Iowa recognized the *Hexagenia* from his Mississippi River experience. He indicated he had found several adults on Cabot side of Sawyer Bay. Inspection the following day, June 23, found hundreds of exuviae/100 m length of shoreline and adults, mostly on shore vegetation. The overall hatch size in Sawyer Bay was estimated as in the several thousands. This hatch originated from the 6/24/2014 stocking of approximately 59,280,000 eggs deployed directly into water column at station H12. It is also notable that the search for young juveniles by Ponar grab on 11/4/2014 revealed no specimens even though the 2016 adult hatch was successful. An additional 20,050,000 eggs were stocked in Sawyer Bay at station H12A in July 2016. This stocking will confound the presumed 2018 hatch as to what might be a natural filial cohort produced from the 2016 adults or from the 2016 stocking.

Station H13, Little Sturgeon Bay. Approximately 10,000,000 eggs were stocked on August 27, 2014 and another 23,000,000 eggs stocked on June 29, 2015. Adult exuviae were collected at this station

in 2016, June (1/100 m), July (7/100 m), and August (2/100 m). No others were observed by kayak surveillance. These few specimens were probably derived from the 2014 stocking.

Station H14, Bullhead Point. No nymphs or adult *Hexagenia* were found in June, July, and August 2016 inspections. 29,000,000 eggs were stocked on August 24, 2014, and thus an adult hatch was expected in 2016; however, none were found. In 2017 a single individual imago was found at Bullhead Point.

“Failure” Sites. The station adjacent to the navigation channel (station Hx15) that had the historical largest populations produced no nymphs, subimagos, or imagos. The search areas were confined to two Coast Guard navigational buoys and the water surface. It is possible that a small hatch did occur, but the adults swarmed along shore some distance from the site. Station Hx11 was located northwest of the ship channel. This was a 1-meter deep site that became heavily choked with macrophytes during summer and is suspect of severe dissolved oxygen sags. No nymphs, subimagos were found. The Oconto River estuary (Station HR9) and Menominee River estuary (Station HR10) have refugium river populations that were indistinguishable from those stocked.

Relative to the number of eggs stocked, only small *Hexagenia* hatches were observed. One critical hindrance during the adult emergence periods was the inability to be at distant sites at the same time or even being at a particular site during the main hatch. For instance, the Sawyer Harbor 2016 hatch that was quantified above was not the main hatch. The main hatch had apparently occurred several days earlier (based on residents’ comments and floating exuviae) when no one was on site. With limited personnel to adequately cover the distances and time frames, camera technology placed at critical points could have been a considerable aid in catching the adult hatches. Another related problem was trying to quantify the adult hatches that did occur, e.g., Sawyer Harbor 2016, because of the mayfly’s chaotic

aerial flight patterns. This could be addressed using drones that can be carefully flown to reduced or eliminate prop wash that creates even greater turbulent imago flights.

The 2017 observed adult hatches were very light. In Sawyer Harbor only 17 adults were seen at station 12A, 3 floating exuviae and no spent subimago exuviae on vegetation. At Bullhead Point in Sturgeon Bay, a single adult was found (on the boat). None were observed at the other stations. While a disappointing hatch, it is possible that a larger main hatch was missed. Alternatively, because of the life cycle prospect of ~24 or ~12 months, or alternation between the two, the 2015 eggs could have been part of the 2016 adult hatch. Or, the 2017 hatch could have been a failed year class.

Conclusions

The field studies presented two benthic fauna communities depending on their inhabitation of nearshore water less than two meters or deep water greater than two meters. The shallower, nearshore communities are in better ecological condition with a diverse composite of *Caenis* and *Baetis* mayflies, amphipods, gilled snails, pulmonated snails, odonates, oligochaetes, chironomids, and meiofauna. The nearshore benthic fauna, including in the AOC, were of greater richness and diversity than the deep water benthic fauna. While this is not an unusual arrangement in lakes considering macrophyte and dissolved oxygen distribution, it appears that the deep-water substrate of lower Green Bay has a severely depressed benthic fauna. Although deep water (>2 m) in the south lower bay is potential prime *Hexagenia* habitat, and the historic sites for large *Hexagenia* populations, >500/m², based on our field studies, deeper areas in the south bay do not represent a suitable habitat for stocking *Hexagenia*. There appears to be a gradual improvement of diversity in the central north lower bay relative to the south lower bay; however, the north area was not historically a site with high *Hexagenia* populations. These results dictated that *Hexagenia* egg stocking be done in the shallow areas. Unless the deep-water hypoxia and fluidized substrate situation improves, it is unlikely *Hexagenia* will robustly recolonize to historic lev-

els. However, the nearshore habitats offer good prospect for successful re-ecesis of *Hexagenia* with the probability of nearshore source colonization of the deep waters as bay improvements take hold in the future.

Stocked *Hexagenia* eggs survived and developed through their life cycle at several shallow water sites in lower Green Bay. The egg stocking in several Green Bay shallow, nearshore water areas in 2016 resulted in the first emergence of adult *Hexagenia* since 1955. While the stocking of *Hexagenia* has produced some encouragement, the lag time between first stocking and development of a sustainable population could be many years to decades. Reaching and maintaining reproductive threshold levels for *Hexagenia* populations will determine their future success.

Hexagenia restoration to a self-sustaining level will have broad management implications. *Hexagenia* is well documented (Great Lakes Fishery Commission, archive search) as an important forage base for game and native fish species. Loss of the *Hexagenia* mayfly population, once prominent in Green Bay, may have depressed fisheries production via loss of a major benthic deposit feeder forage base. The Great Lakes Fisheries Commission's publication has numerous historical and recent accounts of the dietary use of *Hexagenia* by many fishes of the Great Lakes. For example, the importance of burrowing mayflies as a food source for percids has been well documented (Forage Task Group 2014, 2001; Ritchie and Colby 1988; Hayward and Margraf 1987; Scott and Crossman 1973). Hypoxia has eliminated much of the acceptable benthic habitat occupied by mayfly nymphs over their 2-year life cycle, especially in the middle long-axis of the bay.

Hexagenia as an invertebrate forage base extends the spatial range of fish feeding onto mud flats of shallow bay areas, that presently only offer primarily chironomid midge larva as a food source. Fish traditionally fed on mayfly nymphs on the mud flats and on emerging subimagos, and many other wildlife species such as birds, amphibians, reptiles, and small mammals fed exclusively on terrestrial

seeking subimagos and adults. For many *Hexagenia* predators coming into reproductive periods or emerging from winter, the timing of acquiring high value abundant meals synchronized with mayfly emergence can provide a needed energy source that can enhance reproductive success and fecundity.

Acknowledgements

We acknowledge Don Szamania, Kim Weckerly, and Pat Anderson for their assistance and Captain Greg Stamatelakys, Geof Anderson for their valuable assistance and time aboard the R/V Neeskay. This research was supported by grants from the National Science Foundation REU Program, the Sustain Our Great Lakes/National Fish and Wildlife Foundation (0501.14.044507), the NOAA CSCOR Coastal Hypoxia Research Program (NA10NOS4780139), the University of Wisconsin Sea Grant Institute (R/HCE-12), and by the University of Wisconsin-Milwaukee School of Freshwater Sciences.

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Table 1. Station location, depth and sediment percent cohesiveness.

Station Number	Decimal Degrees		Depth M	Spike Test % Cohesive
	Longitude	Latitude		
0	88.005	44.555	1.1	67
2(Hx15)	87.941	44.607	6.4	33
3	88.005	44.607	1.4	100
3A	87.990	44.667	1.2	67
5	87.821	44.658	7.1	67
6	87.883	44.658	6.7	33
8	87.758	44.706	8.5	100
9	87.821	44.706	9.2	67
10	87.883	44.706	8.6	67
12	87.758	44.750	10.3	0
16	87.699	44.793	12.4	67
17	87.758	44.794	12.9	33
18	87.821	44.794	11.6	67
20	87.663	44.839	14.1	67
21	87.696	44.839	15.8	100
22	87.758	44.839	13.6	67
24	87.508	44.883	8.9	0
25	87.571	44.883	20.2	50
26	87.633	44.883	21.6	33
27	87.696	44.883	16.5	33
42	87.383	45.021	20.1	0
43	87.446	45.021	31.7	33
44	87.508	45.021	32.6	0
47	87.383	45.067	30.9	0
S1(H14)	87.392	44.840	3.1	100
S2(Hx11)	87.348	44.822	1.1	67
S3(H12)	87.422	44.887	2.4	100
H7	88.007	44.615	1.4	100
H7A	88.011	44.614	2	100
H7B	88.020	44.556	1.6	67
H8	87.990	44.668	2.1	100
H12A	87.429	44.883	1.7	100
H13	87.556	44.847	1.2	100
HR9	87.833	44.892	2.3	67
HR10	87.606	45.097	2.2	100
NC	87.703	44.831	10.8	67
SC	87.882	44.706	8.5	67

Table 2. Diversity changes at Harris (1998) and Kaster (this paper). Stations Harris15 and Kaster5 were near shore. Harris42,60 and Kaster10, Harris30 and Kaster18, Harris45 and Kaster22 were all central bay stations. Arrows beside Kaster 2011 data indicate diversity relative to Harris 1978 data; no change = →, increasing = ↑, decreasing = ↓.

Site	Simpson 1-D	Shannon H'
Harris15	0.503	1.044
Kaster5	0.494→	1.217↑
Harris42	0.671	1.759
Harris60	0.721	2.260
Kaster10	0.633↓	1.512↓
Harris30	0.606	1.61
Kaster18	0.434↓	1.083↓
Harris45	0.564	1.431
Kaster22	0.379↓	0.930↓

Table 3. Meiofauna averages and standard deviations (n=6) for taxon (order) richness, evenness, Shannon diversity, Simpson's index of diversity, and $H' / 1 - D$ at lower Green Bay sample sites. Sites within the Area of Concern are shown in black, while sites outside are in grey. '*' denotes an average significantly lower ($p < 0.05$) than the average for the same metric at Cat Islands, while '**' denotes an average significantly lower than the average for the same metric at Longtail Point.

	Richness (R)	Evenness (E)	Shannon Diversity (H')	Simpson's Index (1-D)	H' / 1-D
Cat Islands					
\bar{x}	3.17	0.92	1.03	0.62	1.67
SD	0.75	0.07	0.232	0.08	0.16
Longtail Point					
\bar{x}	3.67	0.85	1.09	0.61	1.77
SD	0.82	0.08	0.21	0.09	0.11
Littletail Point					
\bar{x}	3.33	0.81	0.97	0.55	1.77
SD	0.52	0.13	0.22	0.12	0.08
Oconto R. estuary					
\bar{x}	1.83* **	0.68	0.47* **	0.32* **	1.48* **
SD	0.41	0.36	0.25	0.18	0.10
Menominee R. estuary					
\bar{x}	3.33	0.88	1.05	0.61	1.74
SD	0.52	0.10	0.15	0.09	0.10

Table 4. *Hexagenia* eggs stocked in July 2014, 2015, 2016, and 2017.

Year	Deployment Site	Wt. (g)	Est. Quantity
2014	Longtail Pt. (H7)	24.518	28,476,000
	Littletail Pt. (H8)	24.982	29,015,000
	Channel marker (Hx15)	25.462	29,573,000
	Sturgeon Bay (Hx11)	24.836	28,846,000
	Sawyer Bay (H12)	25.327	29,416,000
	Sawyer Bay (H12A)	25.725	29,879,000
	2014 total		
2015	Cat Islands (O, H7B)	22.602	26,251,000
	Longtail Pt. (H7)	22.892	26,588,000
	Littletail Pt. (H8)	21.875	25,407,000
	Menominee R. (HR10)	19.958	23,180,000
	Oconto R. (HR9)	21.349	24,796,000
	Little Sturgeon Bay (13)	19.699	22,879,000
	Sawyer Bay South (H12A)	18.531	21,548,000
	Bullhead Point (S1, H14)	19.319	22,464,000
2015 total			193,113,000
2016	Longtail Pt. (H7A)	85.748	99,707,000
	2016 total		
2017	Longtail Pt. (H7A)	123.24	143,300,000
	Sawyer Bay South (H12A)	19.589	22,778,000
2017 total			166,078,000
4-year total			634,098,000

FIGURE CAPTIONS

Fig. 1. Sampling sites in Green Bay, Lake Michigan. Ponar samples = circled numbers. Sites with *Hexagenia* egg stocking and successful adult hatches = H7, H7A, H7B, H12, H12A, H13, H14. Refugia sites with *Hexagenia* egg stocking = HR9, HR10. Sites with *Hexagenia* egg stocking with no adult hatches = Hx11, Hx15. Sites where in situ condo enclosures were deployed = NC, SC. Sites where in situ mesocosms were deployed = circled 0, 3, 3A, and HR9, HR10.

Fig. 2. Oligochaetes (Tubificidae; largely *Limnodrilus hoffmeisteri*) generally increased in number from 1952 to 1969, then decreased from 1969 to 1978 to 2011. Modified from Harris 1998 and Howmiller 1971.

Fig. 3. Chironomidae density has remained about the same in the south lower bay but decreased in the north lower bay from the pre-1970 period to 2011. Modified from Harris 1998 and Howmiller 1971.

Fig. 4. Tanypodinae 1952 and 1969 (pre-1970) of (A) *Tanytarsus* and (B) *Procladius* showing a considerable decrease in (C) 2011 for Tanypodinae, a largely predatory subfamily of Chironomidae. 5A,B modified from Howmiller 1971.

Fig. 5. Sphaeriidae decreased from A) 1952 and 1969 to B) 2011. A) modified from Howmiller 1971.

Fig. 6. Abundance (m^2) and diversity of Chironominae, Tanypodinae, Tubificidae, and Nematoda at station 6, 17, and 26. Diversity increases from south station 6 to north station 26 largely due to higher density of nematodes in the south. Organic carbon data from Klump et al., this issue.

Fig. 7. A) Water content of central-bay stations (2,3,4,5,6) and nearshore stations. B) Average sized sediment particles.

Fig. 8. Total meiofauna population densities. Error bars represent standard error of the mean. Darker bars represent sites within the Lower Green Bay and Fox River Area of Concern. P-values denote significant differences ($p < 0.05$) in population densities between samples obtained at the two sites at the ends of each bar.

Fig. 9. *Hexagenia* viability. (A) Viable (including dormant); (B) Not viable; (C) Hatched (empty) and neonates (right).

FIGURE 1

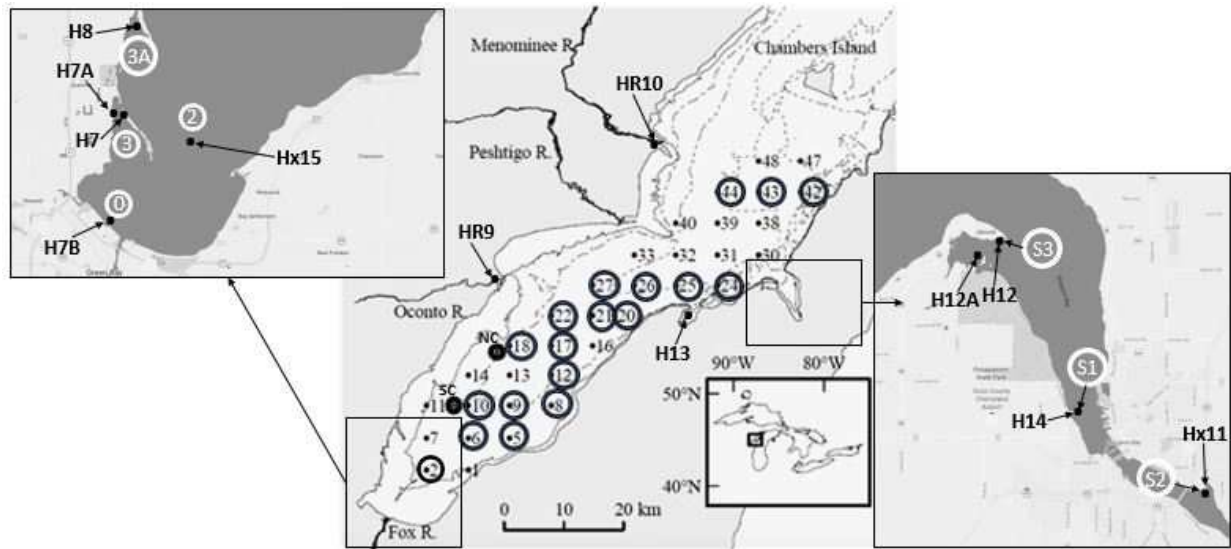


FIGURE 2.

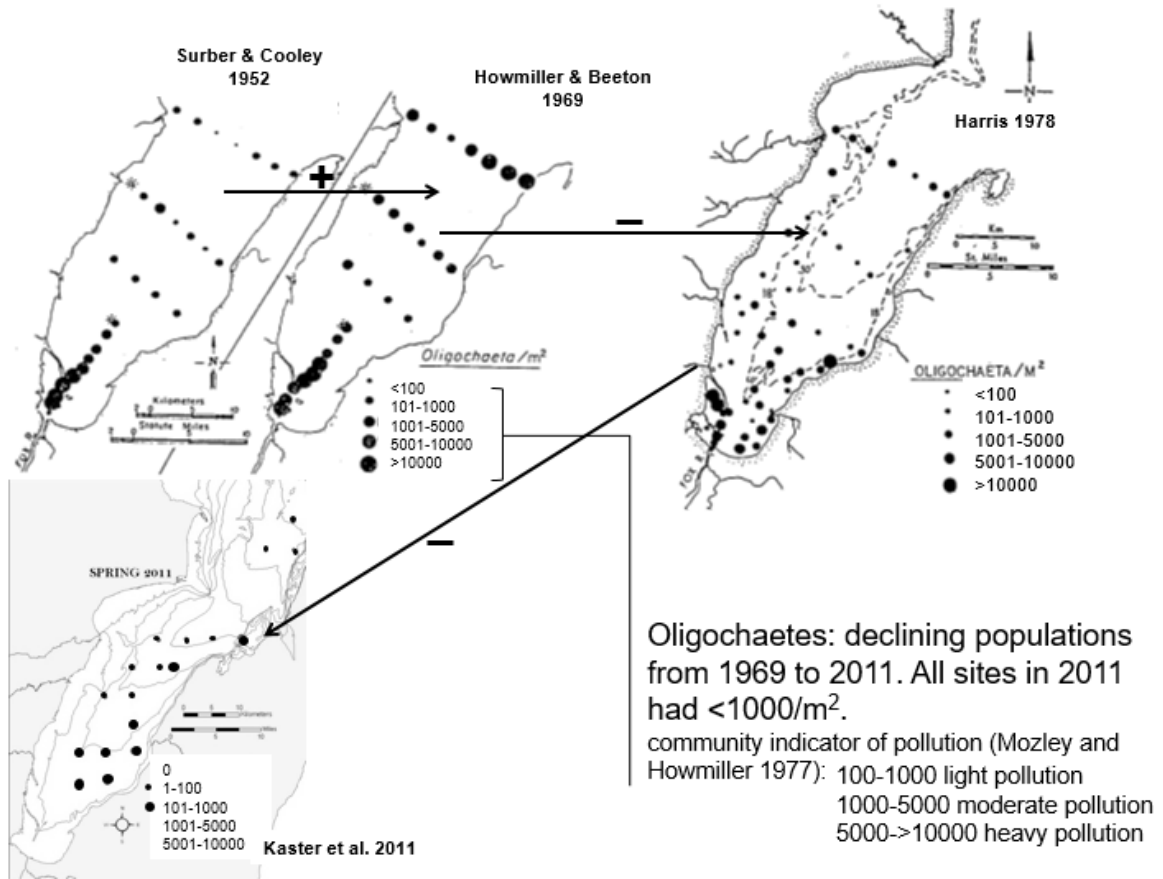
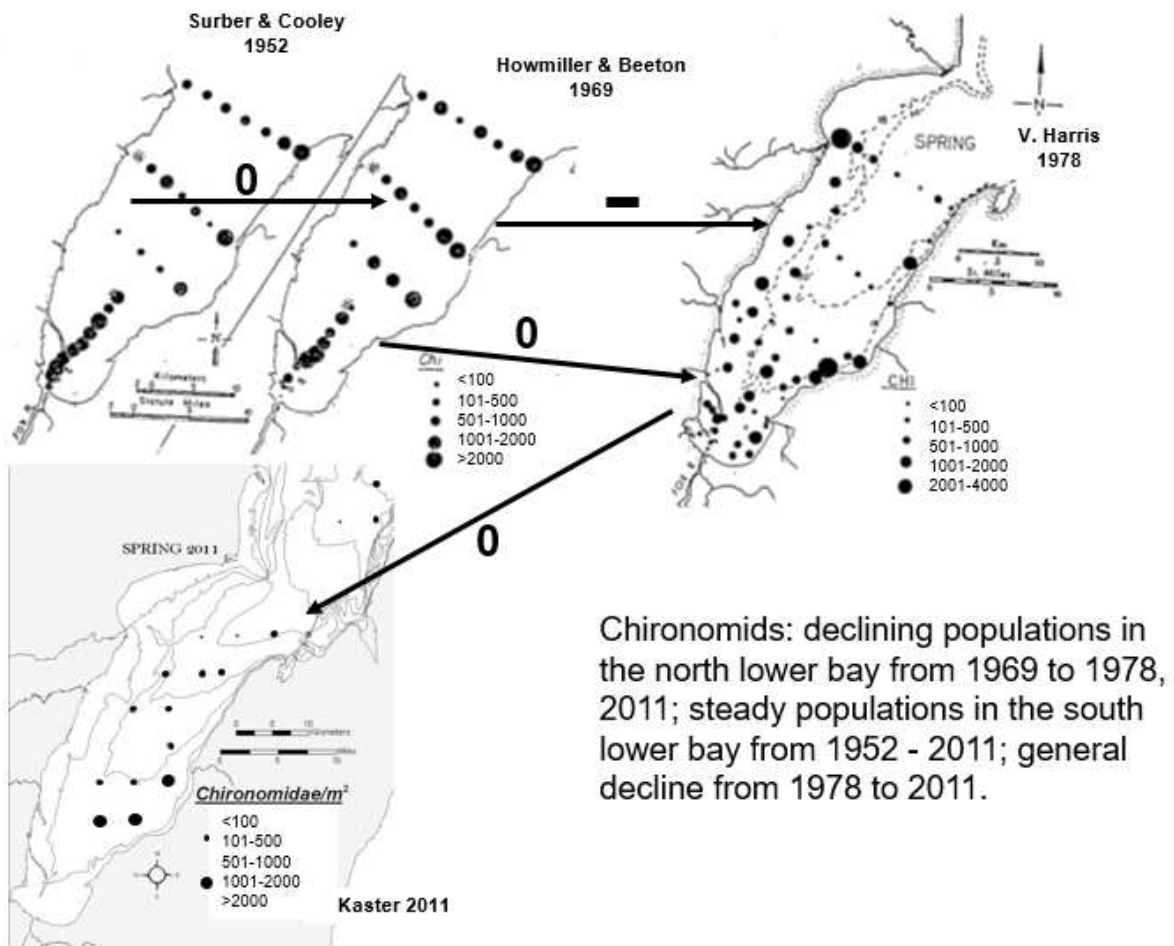


FIGURE 3.



Chironomids: declining populations in the north lower bay from 1969 to 1978, 2011; steady populations in the south lower bay from 1952 - 2011; general decline from 1978 to 2011.

FIGURE 4.

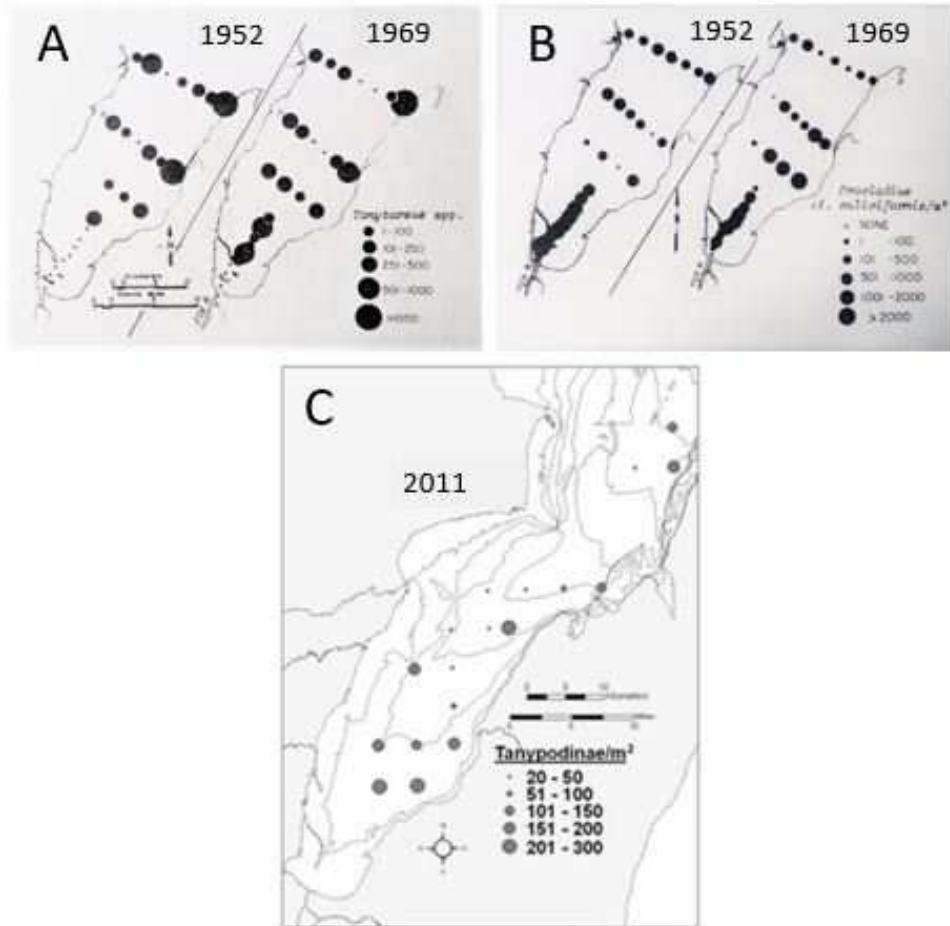


FIGURE 5.

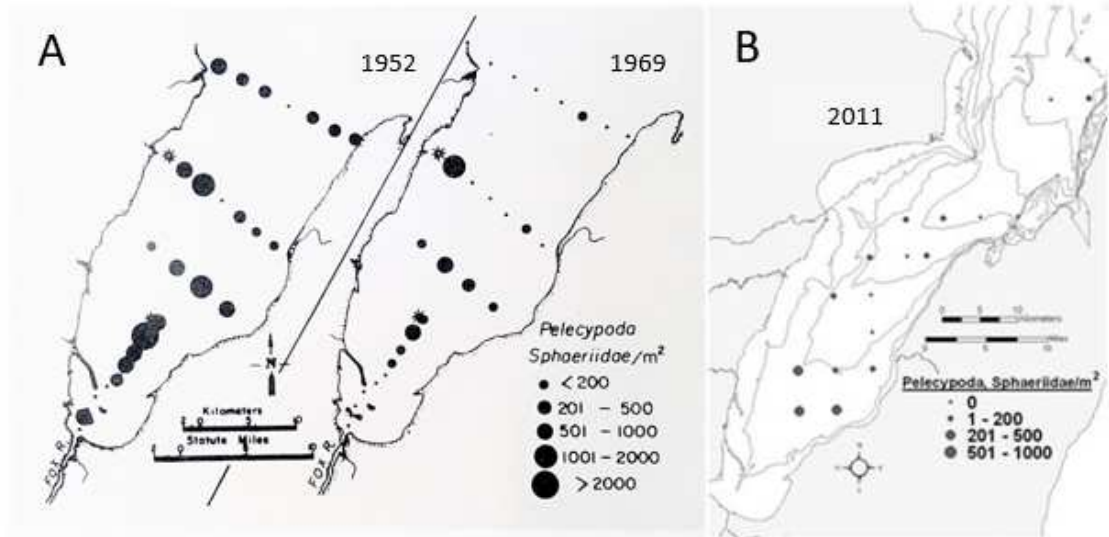
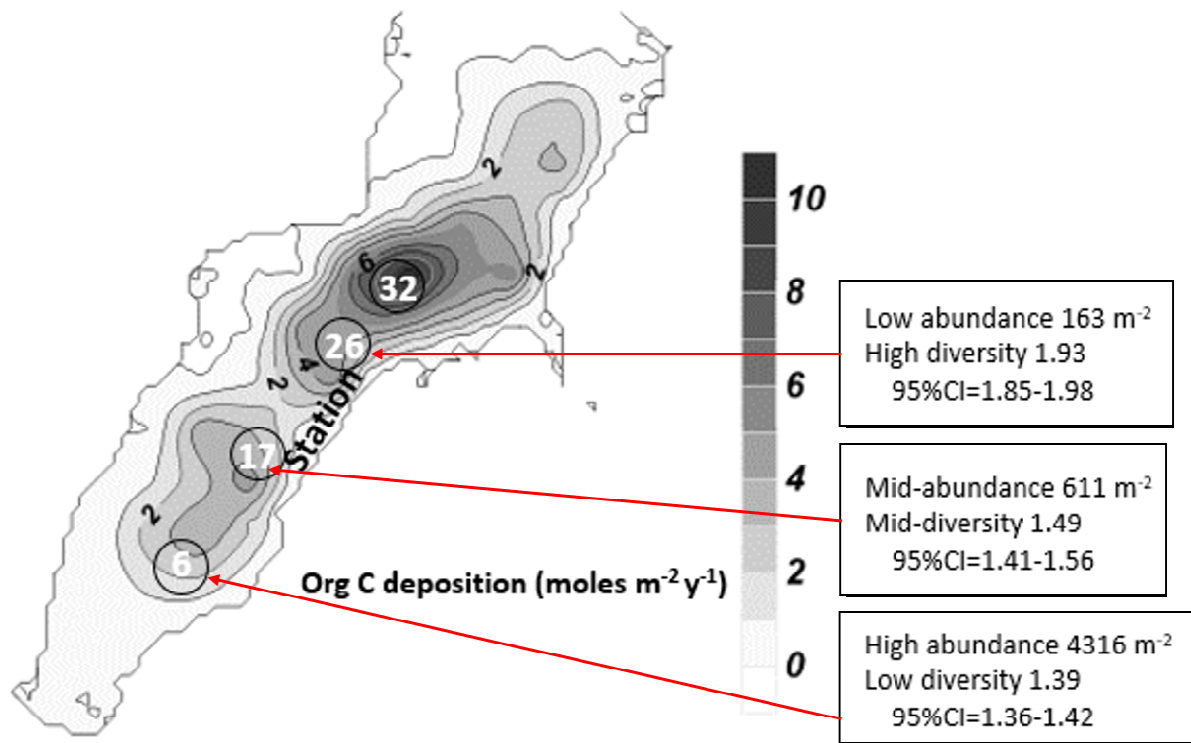


FIGURE 6.



	Station			
Taxon	6	17	26	32
Chironominae	1148	138	26	0
Tanypodinae	203	33	39	0
Tubificidae	269	66	59	0
Nematoda	2696	374	39	0

FIGURE 7.

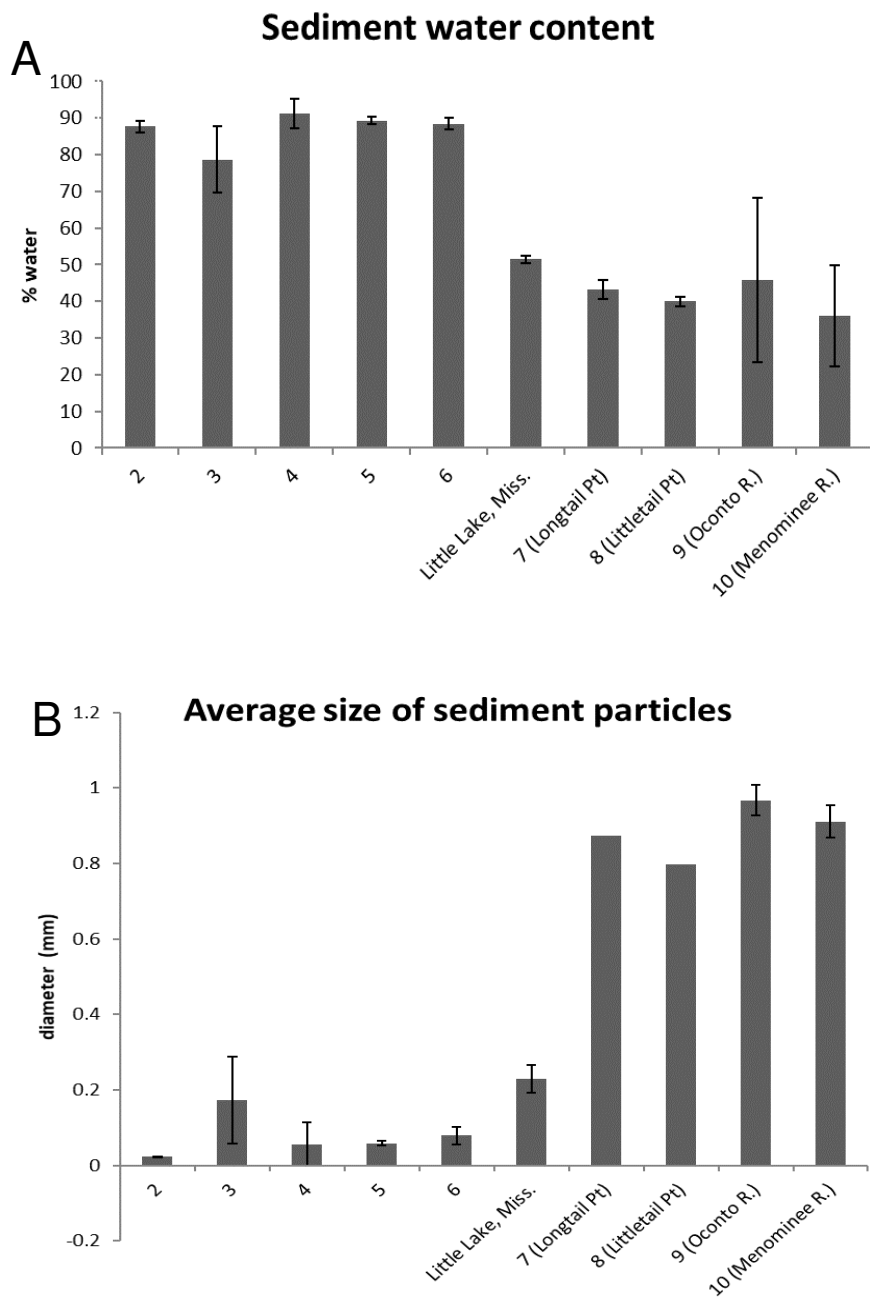


FIGURE 8.

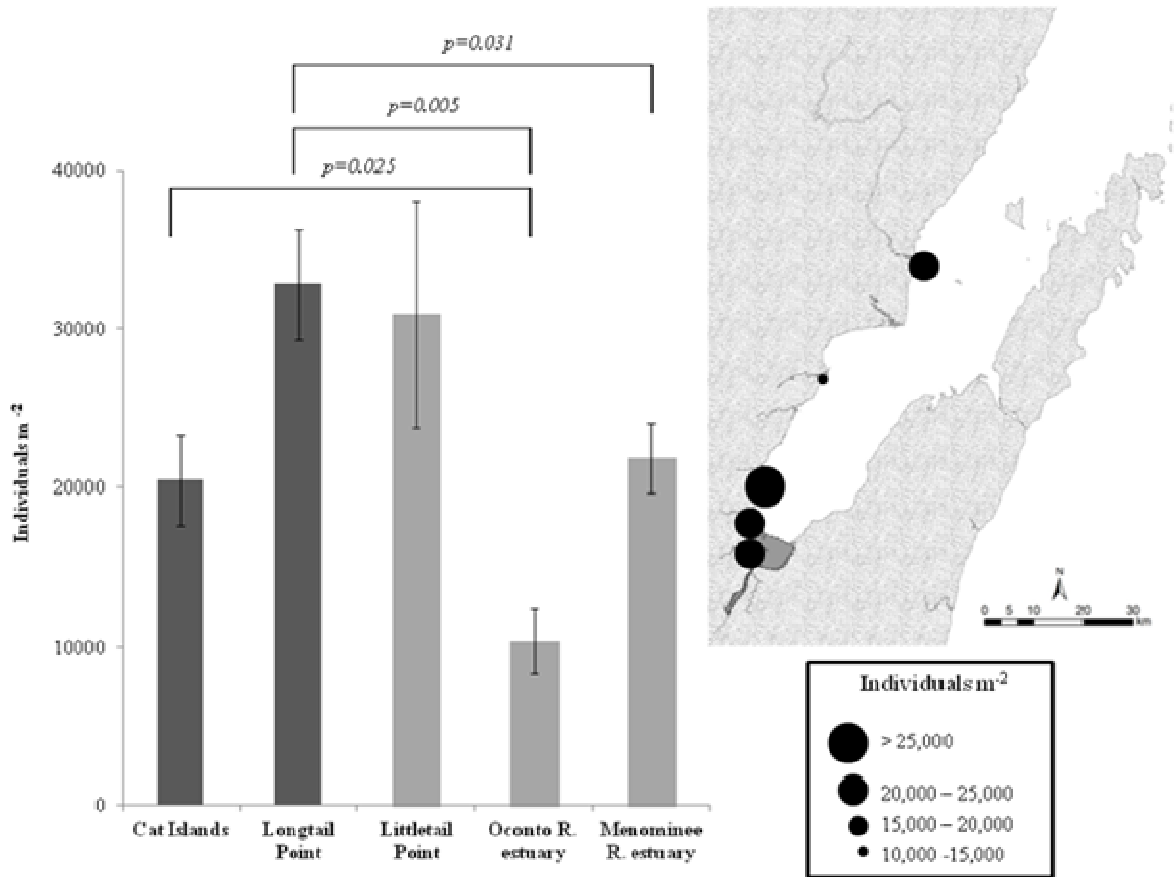


FIGURE 9.

