August 2016

Assessment of Benthic Habitat Quality in Lower Green Bay, Lake Michigan with Special Regard to Potential Hexagenia Recolonization

Christopher Michael Groff
University of Wisconsin-Milwaukee

ASSESSMENT OF BENTHIC HABITAT QUALITY IN LOWER GREEN BAY, LAKE MICHIGAN WITH SPECIAL REGARD TO POTENTIAL *HEXAGENIA* RECOLONIZATION

by

Christopher M. Groff

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Freshwater Sciences and Technology

at

The University of Wisconsin-Milwaukee

August 2016

ASSESSMENT OF BENTHIC HABITAT QUALITY IN LOWER GREEN BAY, LAKE MICHIGAN WITH SPECIAL REGARD TO POTENTIAL *HEXAGENIA* RECOLONIZATION

by

Christopher M. Groff

The University of Wisconsin-Milwaukee, 2016
Under the Supervision of Professor Jerry L. Kaster

With environmental remediation in the Great Lakes, *Hexagenia* have recovered or are recovering in systems from which they were once extirpated. An active *Hexagenia* recovery does not appear to be taking place in lower Green Bay. This study first examines the highly fluidized nature of lower Green Bay sediment as a possible cause for their lack of recovery due to nymphs' potential inability to construct and maintain burrows essential to the completion of their life cycles. *Hexagenia bilineata* nymphs collected from the Upper Mississippi River were distributed into oxygenated aquaria containing substrates from lower Green Bay or the Upper Mississippi River collection site. Fluidized lower Green Bay sediment did not appear to hinder *H. bilineata* survival, growth, production, or biomass turnover in a laboratory setting. These metrics were, in several cases, greater in lower Green Bay substrates compared to control substrates from the nymph collection site. *Hexagenia* egg hatch and young nymph survival in lower Green Bay, tested in situ by artificially stocking eggs collected from adults emerged from western Lake Erie, were shown to be possible, as nine live nymphs ranging from 2-7 mm were recovered near egg stocking sites within one year of stocking. Additionally, meiobenthos, a group suggested to respond negatively to organic pollution, were sampled at several lower Green Bay sites. Densities of Ostracoda, Copepoda, and total meiobenthos, as well as taxon (order) diversity (Simpson's Index, Shannon-Wiener Index, richness, and evenness) were compared between sites within and outside the Lower Green Bay and Fox River Area of Concern (AOC). Results showed that densities and diversity were not significantly

lower within the AOC ($p < 0.05$). Densities were often greater at sites within the AOC, and diversity was relatively consistent between sites. Overall, the results of this study may suggest potentially higher benthic habitat quality in lower Green Bay than was initially expected.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank first and foremost, Dr. Jerry Kaster for his invaluable assistance and guidance during my time as a graduate student at the School of Freshwater Sciences and in my final year as an undergraduate. On one occasion as an undergraduate, I asked him "If you can think of one aspect of your career you've enjoyed the most, what would it be?". His answer: "The ability to explore". It is safe to say this ideal will continue to resonate with me throughout my own career, wherever it may lead.

Additionally, I would like to express thanks to Dr. Val Klump and research specialists Don Szmania, Kim Weckerly, and Pat Anderson, Captain Greg Stematelakys and the crew of the research vessel Neeskay, School of Freshwater Sciences graduate students Dylan Olson, Shelby LaBuhn, and Steve Devilbiss, and to Research Experience for Undergraduates (REU) student Suniel Iyer for their assistance in various aspects of field and lab work involved in this thesis study. Completing this study would have undoubtedly been much more difficult without the help of individuals like these. I would also like to thank the National Fish and Wildlife Foundation (NFWF) for their funding in support of my research.

Lastly, I would like to thank Drs. Val Klump and Jim Waples for a wonderful job in leading the Quantitative Freshwater Analysis course I took during my first semester as a graduate student. The course could have easily been re-titled "Lessons in Repeated Failure", as it essentially addressed solving seemingly impossible problems. The degree of creativity allowed in the course and the confidence I gained in regard to addressing research questions not only aided greatly in my work on this thesis project, but I am sure will apply time and time again in my career. Thanks for teaching us to make sausage, guys.

CHAPTER 1

Survival, Growth, and Production of *Hexagenia bilineata* Mayflies in Fluidized Sediment from Lower Green Bay, Lake Michigan

Introduction

As the largest freshwater estuary in the Laurentian Great Lakes, Green Bay is a relatively shallow and highly productive branch of Lake Michigan (Klump et al. 1997). As in other large bays and estuaries in the Great Lakes, it has been marked over the past century by chemical contamination and nutrient loading. Excessive primary production in the water column has driven up biological oxygen demand at the benthos, and thus frequent seasonal hypoxic and anoxic conditions have been common in the lower bay during stratification in the summer months (Conley 1983, Kennedy 1982, Klump et al. 1997, Qualls et al. 2009). I suggest that the high volume of organic matter accumulating on the benthos is not fully processed in its entirety, resulting in a highly fluidized "sludge-like" gyttja substrate which can be observed across the majority of the open water benthic habitat of the lower bay. This prospect may also suggest inadequate zoobenthic bioturbation reworking relative to the rate of pelagic-benthic coupling of authigenic deposition. Moreover, large areas of fluidized gyttja may increase benthic-pelagic nutrient regeneration, perpetuating primary production. This positive feedback nutrient loop may accelerate total nutrient availability even as allochthonous inputs are abated. Zoobenthic reworking by species like *Hexagenia* represents a forceful process that could break this nutrient loop.

Organic pollution over the past century has had adverse effects on population of benthic invertebrate fauna of lower Green Bay (Howmiller and Beeton 1971, Harris 1998, Schloesser 2009). Macroinvertebrates have for decades been used in several metrics as indicators of the biological quality of ecosystems (Cairnes and Pratt 1993, Hilsenhoff 1988, Howmiller and Scott 1977, Resh et al. 1995, Schloesser and Nalepa 1996). Mayflies of the genus *Hexagenia*, and especially *H. limbata*, widely recognized for their comparative intolerance of habitat degraded by organic pollution (Bauernfeind and

Moog 2000, Fremling and Johnson 1990, Fremling 1989, Hilsenhoff 1988, Reynoldson et al. 1989), were once abundant to the point of nuisance in lower Green Bay. A decline was noted in 1938 (Wisconsin State Committee on Water Pollution 1939), and the last officially reported specimen occurred in 1955 (Balch et al. 1956), after which *Hexagenia* (primarily *H. limbata*) was considered locally extirpated. Other comparable Great Lakes sites saw similar declines or extirpations along the same timescale, but more recently their recovery has been documented. In Lake Erie's western basin, nymphs were found sporadically in very low densities prior to the mid 1990s, when their population experienced a near 40-fold increase in mean density between 1993 and 1997 (Madenjian et al. 1998, Schloesser 2000). This number proved to be highly variable over the next few years, but still remained comparable to densities prior to their relative disappearance in the 1950s (Bridgeman et al. 2006, Krieger et al. 2007, Schloesser and Nalepa 2001, Schloesser et al. 2000). Saginaw Bay has more recently seen a notable recovery of *Hexagenia.* Sampling in 2010 and 2012 (Siersma et al. 2014) recorded both consistently higher densities and greater distribution compared to data reported between 1965 and 2001. The same study also found that distribution patterns were again consistent with historic 1950s distributions.

Documented reports of *Hexagenia* occurrence in lower Green Bay however, have to date been few. Cochran (1992) collected 13 *H. bilineata* adults from the lower Fox River in 1991. A single nymph was found during an educational sampling activity aboard the R/V Jackson near the Green Bay Metropolitan Sewerage District (GBMSD) outfall (Lower Green Bay and Fox River Area of Concern Remedial Action Plan 2013). An individual adult was observed aboard the R/V Neeskay (UW-Milwaukee School of Freshwater Sciences) during the summer of 2012 (Kaster, J.L., University of Wisconsin-Milwaukee School of Freshwater Sciences, personal communication, July 2013). During the same summer, the United States Geological Survey (USGS) encountered 8 nymphs while taking benthic samples near the mouth of the lower Menominee River and another 8 in a similar location on the Oconto River (extrapolating to 51 individuals m^{-2} at both sites) (Eikenberry et al. 2014). Approximately 50 *Hexagenia spp.* (adults) attracted to shore lights were found at Riley's Bay on the western shore of lower

Green Bay in summer 2014 (Janssen, J. University of Wisconsin-Milwaukee School of Freshwater Sciences, personal communication, 2015). Two *Hexagenia spp.* (adults) were also observed during the summer of 2015 near the DePere dam on the lower Fox River, which empties into Green Bay (Harris, H. J., University of Wisconsin-Green Bay, personal communication, July 2015). In light of the sparse observations of *Hexagenia* in the lower Green Bay area, the population does not appear to be actively recovering. Simply aiming for a specific population density target (i.e. as described in lower Green Bay and Fox River Area of Concern Remedial Action Plans) may alone be insufficient in assessing the state of benthic habitat as measured in regard to *Hexagenia*. It may be useful to focus instead on determining their ability to tolerate specific lower Green Bay conditions that may still inhibit a successful re-colonization, e.g., suitable substrate and/or inadequate oxygen at the benthos.

Hexagenia nymphs must construct and maintain u-shaped burrows in the substrate in order to complete their life cycles. Since burrows dip well beneath the oxidized layer on the sediment surface, burrowing insects must continually irrigate their burrows to ensure an adequate supply of oxygenated water (Wang et al. 2001), as well as to aid in accumulating food particles and removing waste (Kristensen 1988, Riisgard 1991). These processes can only occur if burrows are able to remain structurally stable for extended periods of time. Frequently collapsing burrows eventually pose an energetic imbalance for *Hexagenia* to adequately maintain a ventilated burrow (Kaster, J.L, personal communication based on his communication with Dr. Cal Fremling, 1980). The highly fluidized substrate present in much of lower Green Bay's benthic habitat may inhibit burrow construction and/or maintenance, thus limiting *Hexagenia*'s ability to complete life cycles and reproduce. My observation of *Hexagenia bilineata* nymphs present at a site with an observably fluid substrate in the Upper Mississippi River backwaters ("Little Lake") during July 2014 suggested that *H. bilineata* (as opposed to *H. limbata*, the primary *Hexagenia* species historically present in Green Bay) is successful at constructing and maintaining burrows in fluidized substrates, given their high density at the Upper Mississippi River site (1110 m^{-2}, n=12 Ponar grab samples). This may suggest that *H. bilineata* is a better candidate than *H. limbata* for

initial stocking in fluidized sediments as a predecessor to *H. limbata* re-colonization. In Lake Erie, Bustos and Corkum (2012) suggested that *H. rigida* was a predecessor to *H. limbata* re-colonization. These three *Hexagenia* species coexist in varying dominance in habitats across the Great Lakes region (Edsall et al. 2001, Masteller and Obert 2000), and primarily vary in terms of size (*H. biliniata* are very slightly larger) (Fremling 1960). This study tested *Hexagenia bilineata*'s ability to survive and grow in highly fluidized sediment collected from lower Green Bay in an oxygenated microcosm setting. Estimates of secondary production and biomass turnover were also made.

Methods

Hexagenia Nymph and Sediment Collection

Hexagenia nymphs were collected from the Upper Mississippi River site, "Little Lake" (44° 9.435'N, 91° 47.485'W), near Fountain City, WI on October 22, 2014. On a prior trip to the site during July of the same year, *H. bilineata* nymphs were found in high abundance in relatively fluidized substrate. A small motor boat was launched at Merrick State Park (WI), and 12 petite (6x6 inches; ~15x15 cm) Ponar grabs were taken at a location based on observations of mayfly burrow holes in clearings between macrophytes. Each grab was sieved through 1/16" (1.6 mm) nylon mesh, and nymphs were counted and transferred to a portable, aerated cooler containing water from the same area and small fragments of nylon mesh for structure (to reduce a 5-hour transportation stress for nymphs). Sediment from the Little Lake nymph collection site was collected by petite Ponar grab and placed in an additional cooler. Fluidized sediment from central lower Green Bay (44° 48.092'N, 87° 44.144'W) was collected via standard Ponar grab (9x9 inches; ~23x23 cm) on October 15, 2014. All sediment was stored at 4°C until its use in the experiment.

Experimental Design and Procedure

Seven standard 10 gallon (37.9 L, 50.8*l* x 25.40*w* x 30.48*h* cm) glass aquaria were used in this experiment, which was initiated October 23, 2014. Aquaria were loaded with approximately 8 cm of sediment (for a total sediment volume of 0.01 m^3, or surface area of 0.13 m^2 per aquaria). Five aquaria contained lower Green Bay sediment, and the remaining two contained sediment collected from Little Lake, Upper Mississippi River. Percent water content, grain size fractions, porosity, and percentages of nitrogen, total carbon, and organic carbon were calculated for both lower Green Bay and Upper Mississippi River sediments. Water content was obtained by weighing samples before and after drying for 72 hours at 60°C, porosity was calculated based on a sediment density of 2.3 g cm^{-3} , and grain size fractions were calculated using the Bouyoucos hydrometer method (Bouyoucos 1936) (n=3 for both substrates). Nitrogen, total carbon, and organic carbon content were determined via stable isotope analysis. For nitrogen and total carbon, dried and finely ground sediment samples were weighed on a Sartorius Microbalance to 0.001 mg ± 0.002, placed into sealed tin capsules (n=3 for both substrates), and stored in a 96 well plate. Samples were placed into an AS100 auto sampler and combusted at high temperature, converting carbon and nitrogen to N_2 and CO_2 gases, which were separated on a gas chromatography column. Gases were sent to a Finnigan MAT Delta-S Isotope Radio Mass Spectrometer, and data analysis was done using Isodat version 7.2 software. Organic carbon content was determined by acidifying sediment samples in a desiccator containing 6N HCl for 24 hours (n=3 for both substrates) and drying at 60°C for 30 minutes to remove acid fumes, prior to repeating the procedure outlined above.

Sediment was sieved through 1/16" (1.6 mm) nylon mesh prior to being added to aquaria to remove excess *Hexagenia* nymphs and large plant material. Dechlorinated Lake Michigan water was used to fill the aquaria and aeration was provided by air pump and air stones. Window screen tents covered each aquarium to allow emerging winged sub-imagos to cling above the water's surface without being able to escape.

A total of 245 nymphs were separated into three size classes: 10-15 mm in length (n=112), 16-20 mm (n=84), and 21-25 mm (n=49). Lengths were measured to the nearest millimeter from the frontal

process of the head to the end of the abdomen. Individuals from each size class were evenly distributed to seven aquaria, thus each held 16 nymphs of 10-15 mm, 12 nymphs of 16-20 mm, and 7 nymphs of 21-25 mm. The experiment was run for 166 days (October 23, 2014-April 6, 2015). Dissolved oxygen and water temperature readings were taken biweekly using an YSI Digital Professional Series optical meter. Oxygen concentrations of at least 8.0 mg L^{-1} were maintained, as Winter et al. (1996) showed that concentrations above 8.0 mg L^{-1} did not negatively affect *Hexagenia* nymph survival or growth in a laboratory setting. Temperature was not controlled, however aquaria were kept at room temperature (~17.5°C) throughout the experiment. Dechlorinated Lake Michigan water was added as needed to compensate for evaporation. Emergences of sub-imagos were recorded throughout the experiment.

Upon conclusion of the experiment, surface water in each aquarium was drained via siphon. Substrates from each aquarium were sieved through 1/16" (1.6 mm) nylon mesh to collect surviving nymphs which were measured to the nearest millimeter. Survival rates were calculated by subtracting the sum of the total live nymphs collected and sub-imagos having emerged from each aquarium from the original number of nymphs placed in each at the onset of the study. Growth rates of surviving nymphs in terms of length increase were calculated using an initial average length of 16.2 mm based on the size class distribution of nymphs initially introduced to each aquarium, and average final lengths for each aquarium. Initial average length and final average lengths for each aquarium were also converted to dry biomass based on *Hexagenia* specific equations from Edsall et al. (1991): lengths in millimeters (MM) were converted to wet weights (W) in grams based on equation 1, and from wet weights to dry weights (D) based on equation 2.

(1) $\ln (W) = 2.82 \ln (MM) - 11.09$

(2) $\ln (D) = -1.5167 + 1.1189 \ln (W)$

In terms of dry biomass, individual growth (ΔD, measured in mg) values were estimated by subtracting the average initial nymph dry weight (10 mg) from the average final nymph dry weights for

each aquarium, and were converted to daily growth rates ($\Delta D/\Delta t$, measured in mg d^{-1}), where t = 166 days. For estimating Production (P), population densities in each aquarium at t = 0 and t = 166 were first converted to density m^{-2} values (n) by multiplying by a conversion factor of 7.75 based on the approximate number of aquariuma areas in one square meter. Production (mg m^{-2} 166 $days^{-1}$) was estimated using the increment-summation method (Benke 1996), where $P = \bar{n}\Delta D$, with $\bar{n}$ being the mean number of nymphs in a particular aquarium, extrapolated to a density m^{-2} basis, between day 0 and day 166, utilizing one sampling interval or 'increment' (the 166 day duration of the experiment). Production values were also converted to daily production rates by dividing by 166 ($\text{Daily } P = \bar{n}\,\Delta D/\Delta t$). Biomass turnover ($P/\overline{B}$) was calculated for each aquarium by dividing total production by mean dry biomass (mg m^{-2}). Water temperature, dissolved oxygen concentration, sediment water content, grain size fractions, porosity, nitrogen, total carbon, and organic carbon contents, survival (%) and length-based growth rates for each aquarium, as well as dry biomass-based growth rates, secondary production, and biomass turnover for each treatment are reported as averages ± standard deviations.

Results

Lower Green Bay substrate averaged 87.6% ± 1.7 water content, 0.0% sand, 90.2% ± 1.9 silt, and 9.8% ± 1.9 clay. Substrate from the Upper Mississippi River averaged 51.4% ± 1.0 water content, 21.5% ± 3.4 sand, 33.3% ± 3.7 silt, and 45.2% ± 1.6 clay. Porosity averaged 2.02 ± 0.04 and 1.18 ± 0.02 in lower Green Bay and Upper Mississippi River substrates, respectively. Lower Green Bay substrates averaged 0.82% ± 0.05 nitrogen, 7.89% ± 0.36 total carbon, and 4.93% ± 0.11 organic carbon. Upper Mississippi River substrates averaged 0.45% ± 0.28 nitrogen, 3.55% ± 0.10 total carbon, and 2.91% ± 0.28 organic carbon. Water temperatures in all aquaria during the experiment averaged 17.4 ± 0.3°C and dissolved oxygen concentration averaged 8.7 ± 0.1 mg L^{-1}.

Several living nymphs were observed on substrate surfaces in aquaria and within burrows adjacent to aquarium walls, and numerous burrow openings were visible on substrate surfaces. Numerous

exuviae were observed, and winged sub-imagos began to emerge on week 11of the experiment. Emergences from aquaria 1-5 (lower Green Bay substrate) were 9, 10, 4, 10 and 8 respectively, and emergences from aquaria 6 and 7 (Upper Mississippi River substrate) were 6 and 3, respectively.

Survival (surviving nymphs + emerged sub-imagos) in lower Green Bay substrates was 93.8%, 59.4%, 75.0%, 68.8% and 78.1% for an average of 75.0% ± 12.7. Survival in Upper Mississippi River substrates was 31.3% and 50.0%, averaging 40.6% ± 13.3. All surviving nymphs (96) aside from seven individuals exceeded the upper limit of the largest size class established at the onset of the study (25 mm), thus growth in terms of length was approximated by subtracting the initial average length of 16.2 mm from the average length of surviving nymphs collected from each aquarium. Net length increases (mm) over 166 days in aquaria 1-5 (Green Bay substrate) were 14.4 ± 3.8 (n=21), 16.2 ± 3.1 (n=9), 13.9 ± 2.9 (n=20), 14.7 ± 1.6 (n=12), and 14.6 ± 2.9 (n=17) for a treatment average of 14.8 ± 0.9, while aquaria 6 and 7 (Upper Mississippi River substrate) yielded averages of 12.6 ± 3.8 (n=4), and 11.1±3.0 (n=13) for a treatment average of 11.8 ± 1.0. Corresponding daily growth rates (mm d^{-1}) in aquaria 1-5 were 0.09 ± 0.02, 0.10 ± 0.02, 0.08 ± 0.02, 0.09 ± 0.01, and 0.09 ± 0.02 for a treatment average of 0.09 ± 0.01. Daily growth rates (mm d^{-1}) in aquaria 6 and 7 were 0.08 ± 0.02 and 0.07 ± 0.02 for a treatment average of 0.07 ± 0.01.

Increases in dry biomass (mg) of individual nymphs over 166 days averaged 36.68 ± 4.27 in aquaria 1-5, and 24.53 ± 4.13 in aquaria 6 and 7. Daily individual biomass increases averaged 0.22 ± 0.03 mg d^{-1} in aquaria 1-5 and 0.15 ± 0.02 mg d^{-1} in aquaria 6 and 7. Total production over 166 days (P) (mg m^{-2}) averaged 7185 ± 716.9 in aquaria 1-5 and 4083 ± 90.97 in aquaria 6 and 7. Daily P (mg m^{-2} d^{-1}) averaged 43.29 ± 4.32 in aquaria 1-5 and 24.60 ± 0.55 in aquaria 6 and 7 (Table 1). Biomass turnover ($P/\overline{B}$) over the 166 day period averaged 1.79 ± 0.44 in aquaria 1-5 and 1.75 ± 0.55 in aquaria 6 and 7 (Table 2).

	Pop. density in aquarium (No. aq.$^{-1}$)	Pop. density (No. m^{-2}) n	Ave. individual dry mass (mg) D	Biomass (B) (mg m^{-2}) $n*D$	Ave. individual growth (mg) ΔD	Ave. individual daily growth (mg) $\Delta D / \Delta t$	Ave. n ($\bar{n}$) (No. m^{-2}) $(n_0 + n_t) / 2$	Production (P) (mg m^{-2}) $\bar{n}\,\Delta D$	Daily P (mg m^{-2} d^{-1}) $\Delta D / \Delta t$
Aq. 1									
t = 0	35	271	10.0	2713	**35.59**	**0.21**	217	**7723**	**46.53**
t = 166	21	163	45.6	7420					
Aq. 2									
t = 0	35	271	10.0	2713	**43.98**	**0.26**	171	**7498**	**45.17**
t = 166	9	70	54.0	3765					
Aq. 3									
t = 0	35	271	10.0	2713	**32.67**	**0.20**	182	**5949**	**35.84**
t = 166	12	93	42.7	3968					
Aq. 4									
t = 0	35	271	10.0	2713	**35.47**	**0.21**	213	**7560**	**45.54**
t = 166	20	155	45.5	7048					
Aq. 5									
t = 0	35	271	10.0	2713	**35.72**	**0.22**	202	**7197**	**43.35**
t = 166	17	132	45.7	6023					
	Ave. ± SD (lower GB substrate):				***36.68 ± 4.27***	***0.22 ± 0.03***		***7185 ±716.9***	***43.29 ± 4.32***
Aq. 6									
t = 0	35	271	10.0	2713	**27.45**	**0.17**	151	**4148**	**24.99**
t = 166	4	31	37.4	1161					
Aq. 7									
t = 0	35	271	10.0	2713	**21.61**	**0.13**	186	**4019**	**24.21**
t = 166	13	101	31.6	3185					
	Ave. ± SD (Upper Miss. R. substrate):				***24.53 ± 4.13***	***0.15 ± 0.02***		***4083 ± 90.97***	***24.60 ± 0.55***

Table 1. Growth (dry biomass) and secondary production calculations for based on the increment-summation method (Benke 1996), where the 166 day experimental period serves as one increment (i.e., total production (P) is measured in mg m^{-2} experimental period^{-1}. Averages and standard deviations (Ave. ± SD) for each treatment are shown in italics. "Aq." denotes one aquarium trial.

	Mean Biomass ($\bar{B}$) (mg m^{-2}) $[(n*D)_0 + (n*D)_t] / 2$	Production (P) (mg m^{-2}) $\bar{n}\,\Delta D$	Biomass Turnover (166 days) $(P / \bar{B})$	Daily Biomass Turnover (mg d^{-1}) $(P / \bar{B}) / 166$
Aq. 1	5066	7723	**1.52**	**0.009**
Aq. 2	3239	7498	**2.32**	**0.014**
Aq. 3	3340	5949	**1.28**	**0.008**
Aq. 4	4880	7560	**2.18**	**0.013**
Aq. 5	4368	7197	**1.65**	**0.010**
	Ave. ± SD (lower GB substrate):		***1.79 ± 0.44***	***0.011 ± 0.003***
Aq. 6	1937	4148	**2.14**	**0.013**
Aq. 7	2949	4019	**1.36**	**0.008**
	Ave. ± SD (Upper Miss. R. substrate):		***1.75 ± 0.55***	***0.011 ± 0.003***

Table 2. Biomass turnover calculations for each aquarium over the 166 day incubation period and daily biomass turnover rates. Averages and standard deviations (Ave. ± SD) for each treatment are shown in italics. "Aq." denotes one aquarium trial.

Discussion

The near 90% water content of the lower Green Bay sediment illustrated its highly fluidized nature in comparison to sediment from the Upper Mississippi River nymph collection site, which was still noticeably fluidized. Upper Mississippi River sediment contained an average of 36.2% less in water content compared to that from lower Green Bay.

The higher growth and production rates observed in lower Green Bay substrates may be related to the higher organic carbon content in these substrates. The link between *Hexagenia* growth and demand for more organic matter-rich substrates has been well documented by Craven and Brown (1969), and Swanson (1967). Average secondary production in lower Green Bay substrates (7185 mg m^{-2}) was notably close to the value reported by Edsall et al. (1991) for *H. limbata* production in Lake Saint Clair in 1986. This is of interest, as *Hexagenia* production in Lake Saint Clair has been documented at approximately three times higher than the highest values reported anywhere in the northern United States and Canada (Edsall et al. 2001). According to their 1991 study, production from April to October 1986 was 9231 mg m^{-2}. Multiplying our production average for lower Green Bay substrates by the proportion of days in their sampling interval to our experiment length (213/166 days) gives a production value comparable to that from their study—9220 mg m^{-2}—potentially indicative of high quality habitat for *Hexagenia* in the Great Lakes region as measured by secondary production (albeit their study describes production of *H. limbata* rather than *H. bilineata*). Biomass turnover was relatively consistent between lower Green Bay and Upper Mississippi River substrates.

Based on these results, *Hexagenia bilineata* did not appear to be limited by the highly fluidized lower Green Bay substrate in regard to their survival, growth, or production. Repeating the experiment with *H. limbata* nymphs may be of value, as their tolerance to fluidized substrate has not been empirically tested, and no literature currently suggests a difference in the two species' ability to construct or maintain burrows in fluidized substrates. Sediment consolidation "spike" tests in 2013 suggested lower Green Bay

sediment may be too fluid for successful *H. limbata* burrow construction based on in situ microcosms inoculated with *H. limbata* eggs (Kaster, J.L. University of Wisconsin-Milwaukee School of Freshwater Sciences, personal communication, 2015). For historical explanation, as a student of Cal Fremling (Winona State University) in the 1960s, Kaster was taught the spike test for assessing mud consolidation by sticking a 0.8 cm-diameter spike into the mud and slowly rotating it while removing. If the hole did not collapse, the mud was determined to be firm enough to support *Hexagenia* nymph's burrowing lifestyle. It is also worth noting that the influence water movement on substrate surfaces was largely absent in the small-scale laboratory setting of this experiment, whereas it potentially represents a substantial variable in the natural environment. Wind and wave action have been shown to significantly disturb and resuspend surface sediments in shallow systems (Bachman et al. 2000, Carper and Bachmann 1984) (e.g., lower Green Bay). This may increase the likelihood of *Hexagenia* burrow collapse and/or decrease nymphs' ability to effectively clear resuspended sediment from burrow openings. *H. bilineata* is generally expected to tolerate current movement of benthic surficial sediment as it is a riverine/backwater species liable to episodic spates. In situ testing of *Hexagenia bilineata* tolerance to fluidized substrates may support our results and eliminate sediment fluidity as a significant factor still inhibiting a large scale re-colonization of *Hexagenia limbata* to lower Green Bay. In Lake Erie, *H. rigida*'s early recolonization dominance may have been promoted by egg banking (Corkum 2010, Bustos and Corkum 2013) or another ecological influence of *H. rigida* by pre-forming or preconditioning a substrate (cf. limnoforming, Kaster and Groff 2014, proceedings of the 2014 Joint Aquatic Sciences Meeting 5-22-2014, http://sgmeet.com/jasm2014/viewabstract.asp?AbstractID=15652) to make it more amenable to the next species sere stage, in this example *H. limbata*. If sediment pre-forming is in play, then the parallel with *H. bilineata* and *H. limbata* could enhance *H. limbata* recolonization.

If fluidized sediment can effectively be eliminated as a factor inhibiting *Hexagenia's* re-colonization of lower Green Bay (i.e. disregarding sediment resuspension due to wind or wave action), inadequate oxygen at the benthos may represent a prominent factor in their continued absence. *Hexagenia*

stress response to low dissolved oxygen is well established (Rasmussen 1988, Reynoldson and Hamilton 1993, Winter et al. 1996). Lower Green Bay continues to experience regional dips in bottom dissolved oxygen below 5 $mg \cdot L^{-1}$ and below 3 $mg \cdot L^{-1}$ in more isolated spots during the late summer (Klump, J.V., University of Wisconsin-Milwaukee School of Freshwater Sciences, unpublished data). The spatial and temporal occurrences of low DO zones do appear to be highly seasonally variable (i.e., local areas with DO levels more suitable for *Hexagenia* survival are maintained through periods of hypoxia in other areas). In conjunction with inadequate oxygen at the benthos, Schloesser et al. (2000) additionally attributed *Hexagenia*'s inability to establish a robust population in western Lake Erie prior to the mid-1990s to a density effect (i.e. Allee's principle: individual aggregation can improve the survival rate of the population (Odum 1971)). Cochran and Kinziger (1997) also cite Allee's principle as possible rational for the insects' lack of recovery in lower Green Bay. It could be reasonably hypothesized that heavy stocking of *Hexagenia* eggs and/or nymphs in strategic locations in Lower Green Bay may aid in overcoming a reproductive density effect. Continued monitoring of bottom dissolved oxygen and in situ studies involving stocked *Hexagenia* would be essential in addressing hypotheses such as these.

References

1. Bachmann, R.W., Hoyer, M.V. and Canfield Jr., D.E. 2000. The potential for wave disturbance in shallow Florida lakes. Lake and Reservoir Management. 16:4, 281-291.

2. Balch, R.F., Machenthrun, K. M., Van Horn W.W., and Wisniewski T.F., 1956. Biological stud. of the Fox River and Green Bay. Wis. State Comm. Water Pollut. Bull. WP102, pp 74, in: Mozley, S.C., & Howmiller, R.P., 1977. Zoobenthos of Lake Michigan. Environmental Status of the Lake Michigan region. 6:56.

3. Bauernfeind, E., & Moog, O., 2000. Mayflies (Insecta: Ephemeroptera) and the assessment of ecological integrity: a methodological approach. Springer Netherlands, pp. 71-83.

4. Benke, A.C. 1996. Secondary Production of Macroinvertebrates, in: Hauer, F.R. and Lamberti, G.A., Methods in Stream Ecology. Academic Press, Inc., San Diego, pp. 557-563.

5. Bouyoucos, G. J., 1936. Directions for making mechanical analyses of soils by the hydrometer method. Soil Science. 42:3, 225-230.

6. Bridgeman, T. B., Schloesser D.W., and Krause A.E., 2006. Recruitment of *Hexagenia* Mayfly Nymphs in Western Lake Erie linked to Environmental Variability. Ecological Society of America. Ecological Applications. 16:2, 601–611.

7. Bustos, C. & Corkum, L. D., 2013. Delayed egg hatching accounts for replacement of burrowing mayflies *Hexagenia rigida* by *Hexagenia limbata* after recolonization in western Lake Erie. J. Gt. Lakes Res. 39, 168-172.

8. Cairns Jr, J., & Pratt, J. R., 1993. A history of biological monitoring using benthic macroinvertebrates. Freshw. biomonitoring and benthic macroinvertebrates, 10, 27.

9. Carper, G.L. and Bachmann, R.W. 1984. Wind resuspension of sediments in a prairie lake. Canadian Journal of Fisheries and Aquatic Sciences. 41:12, 1763-1767.

10. Cochran, P. A., & Kinziger, A. P. (1997). Hexagenia bilineata(Ephemeroptera: Ephemeridae) persists at low levels of abundance in the lower Fox River, Wisconsin. Gt. Lakes Entomologist, 30(3), 89-92.

11. Cochran, P.A., 1992. The return of *Hexagenia* (Ephemeroptera: Ephemeridae) to the Lower Fox River, Wisconsin. Gt. Lakes Entomologist. 25, 79-81.

12. Conley, D. J., 1983. Limnological characteristics of Green Bay, Lake Michigan, May–October, 1980. M.S. thesis. University of Wisconsin-Green Bay.

13. Corkum, L. D., 2010. Spatial-temporal patterns of recolonizing mayflies after a major disturbance. J. Gt. Lakes Res. 36, 338-344.

14. Craven, R. E., & Brown, B. E., 1969. Ecology of *Hexagenia* naiads (Insecta-Ephemeridae) in an Oklahoma reservoir. American Midland Naturalist. 346-358.

15. Edsall, T.A., Manny, B.A., Schloesser, D.W., Nichols, S.J. and Frank, A.M. 1991. Production of *Hexagenia limbata* nymphs in contaminated sediments in the upper Great Lakes connecting channels. Hydrobiologia. 219:1, 353-361.

16. Edsall, T.A., Haas, R.C. and Adams, J.V. 2001. Annual production of burrowing mayfly nymphs (*Hexagenia spp.)* in US waters of Lake St. Clair. J. Gt. Lakes Res. 27:4, 449-456.

17. Eikenberry, B. C. S., Bell, A. H., Burns, D. J., & Templar, H. A., 2014. Benthos and plankton community data for selected rivers and harbors along Wisconsin's Lake Michigan shoreline, 2012 (No. 824). US Geological Survey.

18. Fremling, C. R., 1960. Biology of a large mayfly, *Hexagenia bilineata* (Say), of the upper Mississippi River. Agricultural and Home Economics Experiment Station, Iowa State University of Science and Technology. Vol. 482.

19. Fremling, C. R., & Johnson, D. K., 1990. Recurrence of *Hexagenia* mayflies demonstrates improved water quality in Pool 2 and Lake Pepin, Upper Mississippi River. In: Mayflies and Stoneflies: Life Histories and Biology. Springer Netherlands, pp 243-248.

20. Fremling, C.R., 1989. *Hexagenia* mayflies: biological monitors of water quality in the Upper Mississippi River. J. Minn. Academy of Science. 55, 139-143.

21. Harris, V. A. 1998. Waterfowl use of lower Green Bay before (1977-1978) and after (1994-1997) Zebra mussel invasion. M.S. thesis. University of Wisconsin-Green Bay.

22. Hilsenhoff, W. L., 1988. Rapid field assessment of organic pollution with a family-level biotic index. J. North American Benthol. Soc. 65-68.

23. Howmiller, R. P. & Beeton, A. M. 1971. Biological Evaluation of Environmental Quality, Green Bay, Lake Michigan. J. Water Pollut. Control Federation. 43:1, 123-133.

24. Howmiller, R. P., & Scott, M. A., 1977. An environmental index based on relative abundance of oligochaete species. J.Water Pollut. Control Federation. 809-815.

25. Kennedy, J. A., 1982. Water-mass structure and exchanges in Green Bay, Lake Michigan. M.S. thesis. University of Wisconsin-Milwaukee.

26. Klump, J. V., Edgington, D. N., Sager, P. E., & Robertson, D. M., 1997. Sedimentary phosphorus cycling and a phosphorus mass balance for the Green Bay (Lake Michigan) ecosystem. Can. J. Fisheries and Aquatic Sciences. 54:1, 10-26.

27. Krieger, K. A., Bur, M. T., Ciborowski, J. J., Barton, D. R., & Schloesser, D. W., 2007. Distribution and abundance of burrowing mayflies (*Hexagenia spp*.) in Lake Erie, 1997–2005. J. Gt. Lakes Research. 33, 20-33.

28. Kristensen, E., 1988. Benthic fauna and biogeochemical processes in marine sediments: microbial activities and fluxes. Nitrogen cycling in Coast. Marine Environments. 275-299.

29. Madenjian, C. P., Schloesser, D. W., & Krieger, K. A., 1998. Population models of burrowing mayfly recolonization in western Lake Erie. Ecological Applications. 8:4, 1206-1212.

30. Masteller, E.C. and Obert, E.C. 2000. Excitement Along the Shores of Lake Erie- Hexagenia-Echoes from the Past. Gt. Lakes Res. Review. 51:25.

31. Odum, E. P., 1971. Fundamentals of Ecology, 3rd ed. WB Saunders, Philadelphia, PA. *In* Schloesser, D. W., Krieger, K. A., Ciborowski, J. J., & Corkum, L. D., 2000. Recolonization and possible recovery of burrowing mayflies (Ephemeroptera: Ephemeridae: *Hexagenia spp*.) in Lake Erie of the Laurentian Great Lakes. J. Aquatic Ecosyst. Stress and Recovery. 8:2, 125-141.

32. Qualls, T. M., Harris, H. J., Harris, V. A., & Medland, V., 2009. State of Green Bay. University of Wis. Sea Grant Inst. University of Wisconsin-Green Bay.

33. Rasmussen, J. B., 1988. Habitat requirements of burrowing mayflies (Ephemeridae: *Hexagenia*) in lakes, with special reference to the effects of eutrophication. J. North American Benthol. Soc. 51-64.

34. Resh, V. H., Norris, R. H., & Barbour, M. T., 1995. Design and implementation of rapid assessment approaches for water resource monitoring using benthic macroinvertebrates. Australian J. Ecology. 20:1, 108-121.

35. Reynoldson, T. B., & Hamilton, A. L., 1993. Historic changes in populations of burrowing mayflies (*Hexagenia limbata*) from Lake Erie based on sediment tusk profiles. J. Gt. Lakes Research. 19:2, 250-257.

36. Reynoldson, T. B., Schloesser, D. W., & Manny, B. A., 1989. Development of a benthic invertebrate objective for mesotrophic Great Lakes waters. J. Gt. Lakes Research. 15:4, 669-686.

37. Riisgard, H. U., 1991. Suspension feeding in the polychaete *Nereis diversicolor*. Marine Ecology Prog. Ser. 70, 29-37.

38. Schloesser, D.W., Krieger, K.A., Ciborowski, J.J. and Corkum, L.D., 2000. Recolonization and possible recovery of burrowing mayflies (Ephemeroptera: Ephemeridae: Hexagenia spp.) in Lake Erie of the Laurentian Great Lakes. *Journal of Aquatic Ecosystem Stress and Recovery*, *8*(2), pp.125-141.

39. Schloesser, D. W., & Nalepa, T. F., 2001. Changing abundance of *Hexagenia* mayfly nymphs in western Lake Erie of the Laurentian Great Lakes: impediments to assessment of lake recovery?. International Rev. Hydrobiol. 86:1, 87-103.

40. Schloesser, D. W., Nalepa, T. F., & Mackie, G. L., 1996. Zebra mussel infestation of unionid bivalves (Unionidae) in North America. American Zoologist. 36:3, 300-310.

41. Schloesser, D.W., USGS, Great Lakes Science Center, 2009. *Hexagenia spp*.; Indicator #122. United States Environmental Protection Agency. State of the Great Lakes 2009: 127-132.

42. Siersma, H. M., Foley, C. J., Nowicki, C. J., Qian, S. S., & Kashian, D. R., 2014. Trends in the distribution and abundance of *Hexagenia spp*. in Saginaw Bay, Lake Huron, 1954–2012: Moving towards recovery?. J. Gt. Lakes Research. 40, 156-167.

43. Swanson, G. A., 1967. Factors influencing the distribution and abundance of *Hexagenia* nymphs (Ephemeroptera) in a Missouri River reservoir. Ecology. 216-225.

44. Wang, F., Tessier, A., & Hare, L., 2001. Oxygen measurements in the burrows of freshwater insects. Freshw. Biology. 46:3, 317-327.

45. Winter, A., Ciborowski, J. J., & Reynoldson, T. B., 1996. Effects of chronic hypoxia and reduced temperature on survival and growth of burrowing mayflies, (*Hexagenia limbata*) (Ephemeroptera: Ephemeridae). Canadian J. Fisheries and Aquatic Sciences. 53:7, 1565-1571.

46. Wisconsin Department of Natural Resources (WDNR), 2013. Stage 2 Remedial Action Plan Update for the Lower Green Bay and Fox River Area of Concern. Wisconsin Department of Natural Resource Office of the Great Lakes. P.O. Box 7921, Madison, WI 53707.

47. Wisconsin State Committee on Water Pollution and State Board of Health in collaboration with the Green Bay Metropolitan Sewage Commission, 1939. Investigations of the Pollution of the Fox and East Rivers and of Green Bay in the Vicinity of Green Bay. Wisconsin State Committee on Water Pollution, Madison, Wis.

CHAPTER 2

Hexagenia limbata Egg Hatch and Young Nymph Survival Potential in Lower Green Bay: In Situ Experiments

Introduction

A Remedial Action Plan (RAP) was developed in 1988 for the Lower Green Bay and Fox River Area of Concern (AOC) (WDNR 1988), which included statements regarding the restoration of populations of pollution-sensitive benthic taxa. A target for *Hexagenia* was set in this report at an average of 400-500 m^{-2}. In 2009, several Beneficial Use Impairment (BUI) delisting targets were established (WDNR 2009), among them a 'Degraded Benthos' BUI, which outlined specific benchmarks for delisting. In regard to *Hexagenia*, the target was reduced to and remains at 100-400 m^{-2} (WDNR 2014) based on densities observed in western Lake Erie following their recovery in that system. As mentioned in the previous chapter, reports of *Hexagenia* occurrence in the lower Green Bay area have been sparse, and the population does not appear to be actively recovering. Aiming for a specific population density of 100-400 m^{-2}, as listed in the Degraded Benthos BUI of the AOC RAP, may alone be insufficient in assessing the state of benthic habitat as measured in regard to *Hexagenia* recovery. It is likely useful to focus instead on determining their ability to tolerate current lower Green Bay conditions (which was suggested in the 2013 AOC Remedial Action Plan Update (WDNR 2013)), followed by hypothesizing which factors may still inhibit a successful re-colonization. The previous chapter addressed the potentially adverse condition of fluidized substrate on *Hexagenia bilineata* burrow construction and maintenance in a laboratory microcosm setting. This chapter focuses on attempts to test *Hexagenia limbata* egg hatch and young nymph survival potential within and outside of the AOC, with the hypothesis that hatch and survival is not possible in situ, suggested by the apparent lack in recovery of *Hexagenia* populations in lower Green Bay. Tests were conducted using in situ mesocosms and via direct deployment of large quantities of eggs into the water column. Ultimately, these tests were aimed at addressing the possibility of large-scale egg stocking as a catalyst in *Hexagenia's* recolonization of lower Green Bay.

Methods

Hexagenia egg collection, quantification, and 'control' hatch rate calculation

Eggs were collected from *Hexagenia* sub-imagos and imagos on June 17 and 18, 2014 in Port Clinton, Ohio during early morning hours (approximately 5:00-9:30AM). Locations of collection included a cement pier extending past the mouth of the Portage River into Lake Erie (41°30.983'N, 82°56.200'W), and on the walls and windows of restaurants adjacent to the lakefront (41°896'N, 82°55.700'W)—streetlights along the pier and bright restaurant signs had attracted the insects to these locations during the night. Several gallon-sized plastic zipper bags full of living or recently expired mayflies were obtained. To isolate large quantities of eggs, handfuls or partial bags of mayflies were simply squeezed into a bucket containing water from Lake Erie. Eggs, which sink fairly rapidly, quickly accumulated on the bottom, and the majority of remaining mayfly parts were discarded. The resultant broth (still containing a fair amount of mayfly parts and debris) was sealed in plastic zipper bags and transported on ice back to Milwaukee. There, it was first filtered through 1/8" mesh sieves to remove large debris, yielding a solution consisting almost entirely of water and eggs. This was then poured slowly over finer mesh filters (250µm, 125µm, and coffee filters), isolating clumps of eggs, which resembled fine sand. Eggs were 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.

To quantify the large number of eggs present, a predetermined average wet weight per egg was used. This value was determined by Barbour and Kaster (2012) by weighing three separate 'clumps' of eggs (dabbed lightly with paper towel to remove excess moisture) on a 4-place microbalance. Eggs in each of the three samples were counted, allowing for the calculation of an average mass per egg. The average mass per egg (0.861×10^{-6} g) was used in estimating total numbers of eggs collected and quantities to be deployed at selected lower Green Bay sites.

The procedures outlined above for egg collection and quantification were also employed for eggs collected from Port Clinton on June 24, 2015. Laboratory 'control' hatch rates for eggs collected were also determined, to be used as a standard for comparison of hatching in situ. This involved placing 10-14 eggs (2014) and exactly 10 eggs (2015) each into 10 scintillation vials, filled approximately half way with Lake Michigan water. Vials were checked daily for hatches, and the number of hatches each day was recorded. The total number of hatches in all ten vials was calculated over a 31 day period (2014) and a 45 day period (2015) (daily checking for hatches was concluded when no hatches were observed for 10 consecutive days).

Hexagenia mesocosm experiments and en masse egg deployments

2014

Each of the two mesocosms deployed consisted of six 250 mL mason jars covered with 160 μm mesh (hot glued to the threaded ring of the original mason jar cap with the central lid removed). These were placed in perforated plastic baskets approximately 30 x 20 x 15 cm (*L* x *W* x *H*).

On June 24, 2014, field deployment took place with one mesocosm at Longtail Point (44°36.906N 88°0.417W), and the other at Littletail Point (44°35.571'N, 87°59.438'W) (Fig. 1). A small amount of sediment from each respective deployment site (about 125 mL, or half the volume of one mason jar) was collected via petite (6 x 6") Ponar grab and placed into each jar, along with 100 eggs, which had been previously counted and stored in separate scintillation viles containing Lake Michigan water. Mesocosms were deployed at a depth of approximately one meter at both sites, and small subsurface floats were attached for aid in recovery.

Remaining eggs were weighed on a four-place microbalance and separated into two bottles containing a small volume of Lake Michigan water thus that each bottle held approximately the same quantity of eggs. Based on the average mass per egg of 0.861×10^{-6} g, approximately 28,500,000 eggs were deployed at the Longtail Point site and approximately 29,000,000 at Littletail Point by dumping each

Figure 1. Mesocosm and *en masse* egg deployment sites in lower Green Bay. Site 1: "Cat Islands" (44° 33.144'N, 88° 1.269'W), site 2: "Longtail Point" (44° 36.902'N, 88° 0.425'W), site 3: "Littletail Point" (44° 40.049'N, 87° 59.390'W), site 4: "Oconto River estuary" (44° 53.526'N, 87° 49.983'W), site 5: "Menominee River estuary" (45° 5.820'N, 87° 36.391'W). Dotted line and enclosed shaded region shows the approximate area encompassed by the Lower Green Bay and Fox River Area of Concern.

bottle near the water's surface. Eggs were found to sink rapidly, so a high degree of drift was not assumed to be likely (i.e., they were likely deposited on the sediment surface very near the spot they were deployed into the water column). Egg wet weights and estimated quantities are given in Table 3.

The first mesocosm recovery attempt took place July 29, 2014, however only the unit at Littletail point was recovered (an incubation period of 37 days). Sediment in mason jars from the recovered mesocosm was inspected carefully under a 10x dissecting microscope. 10 petite Ponar grabs each were taken at Longtail Point and Littletail Point and sieved with 0.5 mm mesh immediately in the field. These samples were examined under a 10x dissecting microscope to determine whether or not neonates having hatched from directly deployed eggs were present. Two additional, still unsuccessful attempts to recovery the mesocosm at Longtail Point were undertaken during August and November 2014. On November 4, 2014, 10 additional petite Ponar grabs were taken each at Longtail Point and Littletail Point, again sieved with 0.5 mm mesh and searched in the laboratory under a 10x dissecting microscope for the presence of nymphs.

2015

On March 11, 2015, five petite Ponar grabs each were taken through three holes (approximately 15 m apart) in the approximately 0.5 m ice cover at the Longtail Point site (Kaster, J.L, Klump, J.V. and Szmania, D., UWM School of Freshwater Sciences, personal communication). Prior to filtering through 0.5 mm mesh, the overlying water, or 'supernatant' on each Ponar grab sample (along with some surface sediment) was collected. Supernatants were collected as separate samples in an effort to isolate young nymphs or 'neonates' (which remain largely epibenthic) and to avoid their disfigurement, which may have occurred during the sieving process of samples taken in 2014. 10 Samples were again examined under a 10x dissecting microscope.

New mesocosm enclosures were deployed on June 29, 2015 at the same sites used in 2014 (Longtail and Littletail Points), as well as at the Oconto River estuary (44° 53.526'N, 87° 49.983'W),

Year	Deployment Site	Bottle No.	Wet Wt. (g)	Est. Quantity
2014	Longtail Pt.	1	24.5183	28500000
	Littletail Pt.	2	24.9819	29000000
	2014 total			***57500000***
2015	Cat Islands	1	22.6018	26300000
	Longtail Pt.	2	22.8922	26600000
	Littletail Pt.	3	21.8751	25400000
	Menominee R. est.	4	19.958	23200000
	Oconto R. est.	5	21.3492	24800000
	2015 total			***126300000***
	2-year total			***183800000***

Table 3. Wet weights as recorded by four-place microbalance and estimated quantities of eggs deployed *en masse*.

Menominee River estuary (45° 5.820'N, 87° 36.391'W), and near the Cat Islands (44° 33.144'N, 88° 1.269'W) (Figure 1). Each consisted of a round metal tin approximately 30 cm in diameter containing five 250 mL mason jars with lids again replaced with 160 μm mesh, however each was loaded only with enough substrate to cover the bottom of the jar (as opposed to half the jar, as was the case in 2014). Each jar was inoculated with 100 eggs. Incubation period was increased to 105 days, and recovery attempts took place on October 6, 2015. Each jar recovered from a mesocosm enclosure was placed in a plastic zipper bag with lake water, and placed in a 4°C cooler until inspection under 10x dissecting microscope.

An additional *en masse* deployment of eggs directly into the water column took place on June 29, 2015. Approximately 26,600,000 were deployed at Longtail Point, 25,400,000 at Littletail Point, 25,000,000 at the Oconto River estuary, 23,200,000 at the Menominee River estuary, and 26,300,000 at Cat Islands. Egg wet weights and estimated quantities are given in Table 3. Ten petite Ponar grab supernatant samples were collected at each egg deployment site on October 6, 2015, and three additional petite Ponar grab supernatant samples were collected from the Cat Islands site in November 2015 (Kaster, J.L., UWM School of Freshwater Sciences, personal communication) and examined via 10x dissecting microscope in an effort to recover neonates.

Results

Control hatch rates

Fifteen of 107 eggs hatched within 32 days for a rate of 14% in 2014 (Table 4). Hatches by day are given in Figure 2. Hatches began 21 days post-incubation and continued to day 32, with over half of hatches occurring on day 21. In 2015, 25 of 100 eggs hatched within 45 days for a rate of 25% (Table 5). Hatches by day are given in Figure 3. Hatches began 15 days post-incubation and continued to day 45.

Vial No.	Eggs inserted	No. Hatched	Hatch Rate
1	12	0	0
2	10	1	0.1
3	10	2	0.2
4	14	3	0.21
5	10	2	0.2
6	10	2	0.2
7	10	0	0
8	10	2	0.2
9	11	0	0
10	10	3	0.3
Totals	***107***	***15***	***0.14***

Table 4. Laboratory control hatch rate calculation for eggs collected in 2014.

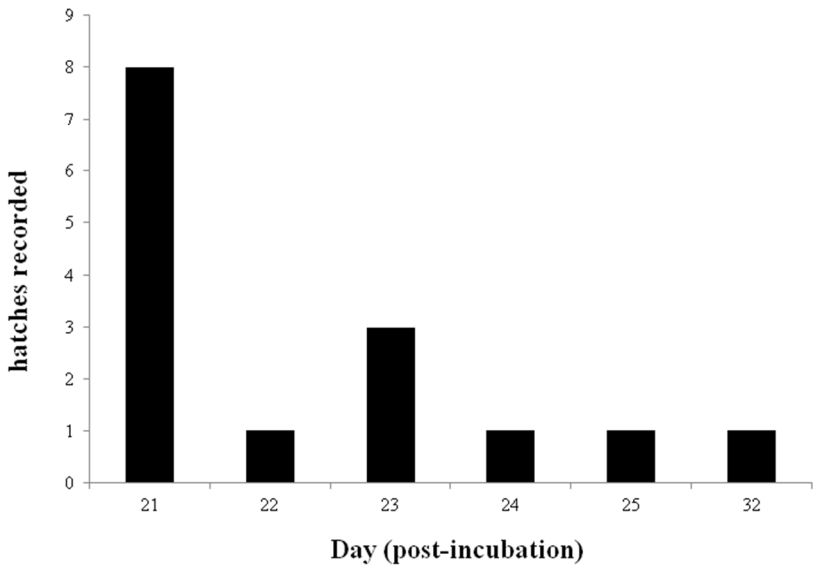

Figure 2. Laboratory control hatches by day for eggs collected in 2014 (number of hatches recorded on a given day are combined values for all ten vials).

Vial No.	Eggs inserted	No. Hatched	Hatch Rate
1	10	4	0.4
2	10	2	0.2
3	10	2	0.2
4	10	1	0.1
5	10	3	0.3
6	10	2	0.2
7	10	4	0.4
8	10	1	0.1
9	10	3	0.3
10	10	3	0.3
Totals	***100***	***25***	***0.25***

Table 5. Laboratory control hatch rate calculation for eggs collected in 2015.

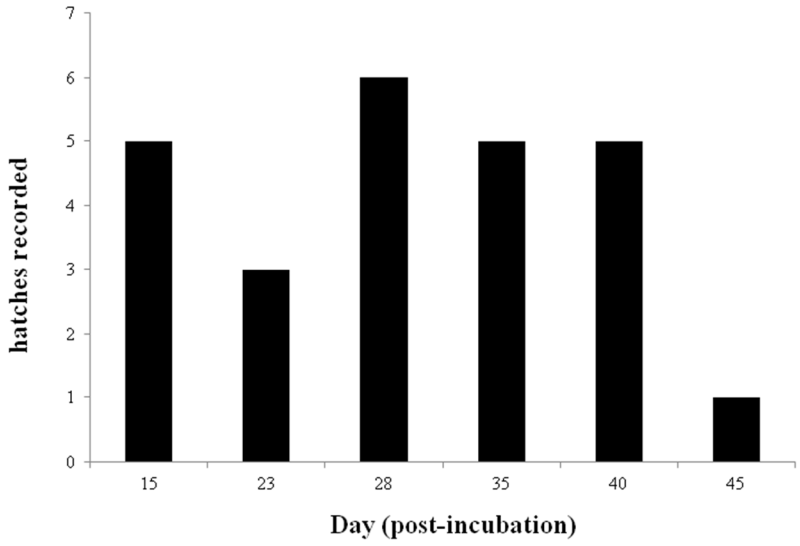

Figure 3. Laboratory control hatches by day for eggs collected in 2015 (number of hatches recorded on a given day are combined values for all ten vials).

Egg hatch and nymph survival in mesocosms

As stated in the methods section, only the mesocosm unit at Littletail Point was recovered in 2014. Two live neonates (~1.5 mm in length) were recovered from one 250 mL mason jar, and the remaining five jars yielded no nymphs. Overall, the two nymphs recovered represented 0.33% of the 600 eggs in the mesocosm. Based on the control hatch rate for eggs collected in 2014, the two nymphs represented 2.4% of potential hatches. All mesocosms recovered in 2015 were covered with several centimeters of sediment and in some cases macrophytes, and no live nymphs were recovered.

Egg hatch and nymph survival from en masse egg deployments

Petite Ponar grab samples sieved with 0.5 mm mesh at Longtail Point and Littletail Point in November 2014 yielded no nymphs. Petite Ponar grab supernatant samples collected through the ice at Longtail Point in March 2015 yielded six "post-neonate" nymphs, all on the order of 2 mm. Supernatant samples taken at Longtail Point in June 2015 yielded one nymph on the order of 7 mm. No *Hexagenia* were found in samples from Littletail Point taken on the same day. No nymphs were encountered in samples collected in October 2015. Two nymphs on the order of 2 mm were encountered in the samples taken at Cat Islands in November 2015. A total of one 7 mm nymph and eight 2 mm nymphs were thus recovered from egg deployment sites (both within the AOC) over the course of this study.

Discussion

Although the number of young nymphs recovered from mesocosms or Ponar grab samples was low, the initial hypothesis that egg hatch and nymph survival would not be possible in lower Green Bay was rejected. Attempts to make data more robust in regard to *Hexagenia* egg hatch and nymph survival potential in situ in lower Green Bay would likely involve improvements to experimental design, as well as to nymph detection and quantification methods for eggs deployed *en masse*.

Based on the failure of the mesocosms deployed in 2015, a better design might be employed for repeating this experiment in the future involving the use of deeper, perforated baskets like those used in 2014. 2015 mesocosms may have sunken into the soft sediments at deployment sites to a degree that sediment was able to be washed from adjacent areas onto the tops of mason jars, limiting oxygen within and thus creating an anoxic environment wherein eggs were unable to hatch or neonates were unable to survive. The solid walls of the metal tins containing mason jars may have also allowed the mesocosms to function as effective sediment traps—sediment washed in may have been unable to escape, as it might have been able to from the perforated 2014 mesocosms. In regard to all mesocosms used in this study (both in 2014 and 2015), the 160 μm mesh used to cover mason jars, though fine enough to contain eggs, may have been too fine to allow adequate transfer of dissolved oxygen from surrounding water into the jars. Low dissolved oxygen was suggested by Barbour and Kaster (2012) to induce facultative dormancy in *Hexagenia* eggs. Sporadic periods of low bottom DO in the surrounding water itself are common during summer months in lower Green Bay (Klump et al. 1997, Klump, J.V. University of Wisconsin-Milwaukee School of Freshwater Sciences, unpublished data 2011-2014). As mentioned in the previous chapter, these periods of low bottom DO may also still play a substantial role in *Hexagenia* populations' apparent lack of recovery in lower Green Bay. In future studies, the use of larger mesocosms (i.e., limnocorrals) may be beneficial. Limnocorrals have been used in many studies to test benthic invertebrates' responses to various environmental factors (Hayne and Ball 1956, Caquet et al. 2000, O'brien et al. 1992). Due to their larger scale compared to mesocosms like those used in this study, better extrapolations of results could likely be made to *Hexagenia* egg hatch and nymph survival in the natural environment, although such extrapolations might be more reliably made on a qualitative basis as opposed to quantitative (Bloesch et al 1988), as environmental differences between the natural environment and the areas within limnocorrals may present restrictions (e.g., magnitude of currents, sedimentation, etc.).

Although we observed eggs deployed en masse to the water column to sink rapidly, eggs and/or recently hatched nymphs may have experienced a higher degree of drift than was expected. The six "post-

neonate" nymphs recovered at Longtail Point in March 2015 were found in samples taken from three separate holes in the ice approximately 15 meters apart. Attempting to collect Ponar grab samples at the exact locations at which eggs were deployed alone was not likely an adequate method for assessing egg hatch or nymph survival potential. Sampling several locations at gradually increasing radii from egg deployment sites may have provided more information regarding the degree of drift of hatched nymphs, and ultimately collecting a greater number of samples may have increased the likelihood of detecting hatched nymphs. The use of Environmental DNA (eDNA) detection methods in freshwater systems has also gained attention in recent years as a potentially valuable tool for the detection of animals present in low densities (Jerde et al. 2011) or those that are rare or threatened (Thomsen et al. 2011). These techniques have largely been applied to fish (e.g., Asian carp, Jerde et al. 2011) or amphibians, which, due to their physiologies, release a comparatively large amount of extracellular DNA into the aquatic environment in comparison to invertebrates (Tréguier et al. 2014). Still, several studies have suggested eDNA to be a useable method for the detection of invertebrates. Goldberg et al. (2013) concluded that the DNA of the invasive New Zealand mudsnail (*Potamopyrgus antipodarum*) could be detected for as few as one individual in 1.5 L of water, and for up to 44 days after the animal was removed. They also confirmed that eDNA could detect the presence of the mudsnail at densities as low as 11 m^{-2} in the natural environment. A 630-base-pair segment of a mitochondrial gene (cytochrome c oxidase 1 (COI)) was sequenced from one individual each of 80 mayfly species obtained in the northeastern United States and Canada to create a species reference profile (Ball et al. 2005). DNA barcodes created by Ball et al. (2005) were able to successfully identify 69 out of 70 additional specimens to species level. Another sequence of the same gene was also used by Elderkin et al (2012) to successfully distinguish between *Hexagenia limbata* and *Hexagenia rigida* in 19 out of 20 cases; the two species can be nearly indistinguishable in appearance. Based on this recent use of eDNA in regard to invertebrate detection and DNA sequencing for *Hexagenia spp*. identification, I suggest that eDNA-based methods might be useful in lower Green Bay to aid in early detection of *H. Limbata* at egg stocking sites. If egg stocking can be used on a

continual basis and on a larger scale, *Hexagenia* presence may also eventually be detected in fish stomach content samples if a significant number of eggs hatch and nymphs survive to suitable prey sizes for fish.

The fact that *Hexagenia* are highly fecund, r-selected species also aids greatly in the potential for artificial stocking to effectively spur their recolonization of ecosystems from which they were extirpated (e.g., lower Green Bay). Each gravid adult female *H. limbata* can harbor up to 8000 eggs (Hunt 1951, Neave 1932). A simplified model might be suggested in which it is assumed that all females produce 8000 eggs. If this is the case, only 0.0125% of eggs laid must survive to become reproducing females in the subsequent generation for the same number of eggs which spawned the first generation to be produced (thus keeping the population at equilibrium at each successive reproductive cycle). Applying this model to the number of eggs deployed at Longtail Point in 2014 (approximately 28,500,000), only 3563 eggs must become reproducing females for 28,500,000 eggs to be laid in the next reproductive cycle. If it is further assumed that only 14% (laboratory control hatch rate) of deployed eggs hatched at Longtail Point in 2014 (3,990,000 hatches), the number of individuals which must survive to become reproducing females in order for 28,500,000 eggs to be laid to produce the subsequent generation is still less than 0.1% (0.089%). Assuming this model, if hatch rates in the ambient environment are similar to my laboratory control hatch rate, and female nymphs survive to become reproducing females at a rate greater than 0.089%, the population would increase in density through successive generations in the absence of significant ecological disturbances. It is also worth noting that *Hexagenia* are capable of parthenogenic reproduction (Funk et al. 2010, Sweeney and Vannote 1987), i.e., viable eggs can still be laid by non-mating females, which may further increase potential recolonization success initiated by artificial egg stocking due to the fact that mating does not necessarily need to occur.

The current minimum target density for delisting the Degraded Benthos BUI of the Lower Green Bay and Fox River AOC Remedial Action Plan regarding *Hexagenia* is 100 individuals m^{-2}. The entire AOC (minus the seven miles of the lower Fox River falling within its boundary) is approximately 34 square miles, or 8.8×10^7 m^2, thus a total of 8.8×10^9, or 8.8 billion *Hexagenia* nymphs would have to be

present if a uniform density of 100 individuals m^{-2} was to be maintained throughout the AOC. Madenjian et al. (1998) calculated an annual *Hexagenia* population growth rate in western Lake Erie between 1991 and 1997 (including a factor for mortality) to be 0.8, or 80%. If the number of eggs stocked at Longtail Point in 2014 (28.5 million) were stocked annually throughout the AOC, and the population increased by 80% each year, the target density of 100 nymphs m^{-2} (8.8 billion nymphs total) could be surpassed within 9 years (the population would increase from 7.2 billion to 13 billion between years 8 and 9). This assumes stocking would be done more or less evenly to ensure an even distribution of eggs throughout the AOC. It should be noted that this heuristic model makes broad assumptions (e.g., a significant portion of lower Green Bay benthic habitat is suitable for *Hexagenia* survival and growth), and numerous biotic or abiotic factors may significantly influence the calculations involved. The collection and stocking of a number of eggs larger than 28.5 million annually is almost certainly feasible however, and given the potential reproductive density effect (Allee's Principal) discussed in the previous chapter, highly intensive stocking may result in population densities approaching the AOC target just a few years after stocking is initiated.

References

1. Ball, S.L., Hebert, P.D., Burian, S.K. and Webb, J.M. 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. Journal of the North American Benthological Society 24:3, 508-524.

2. Barbour, S.A. and Kaster, J.L. 2012. *Hexagenia* eggs enter facultative dormancy in Green Bay benthic enclosures. Research Experience for Undergraduates (REU) research project, UW-Milwaukee School of Freshwater Sciences, June-August 2012.

3. Bloesch, J., Bossard, P., Bührer, H., Bürgi, H.R. and Uehlinger, U. 1988. Can results from limnocorral experiments be transferred to in situ conditions?. Hydrobiologia. 159:3, 297-308.

4. Caquet, T., Lagadic, L. and Sheffield, S.R., 2000. Mesocosms in ecotoxicology (1): Outdoor aquatic systems. In Reviews of environmental contamination and toxicology (pp. 1-38). Springer New York.

5. Elderkin, C.L., Corkum, L.D., Bustos, C., Cunningham, E.L. and Berg, D.J. 2012. DNA barcoding to confirm morphological traits and determine relative abundance of burrowing mayfly species in western Lake Erie. Journal of Great Lakes Research. 38:1, 180-186.

6. Funk, D.H., Sweeney, B.W. and Jackson, J.K. 2010. Why stream mayflies can reproduce without males but remain bisexual: a case of lost genetic variation. Journal of the North American Benthological Society. 29:4, 1258-1266.

7. Goldberg, C.S., Sepulveda, A., Ray, A., Baumgardt, J. and Waits, L.P. 2013. Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). Freshwater Science. 32:3, 792-800.

8. Hayne, D.W. and Ball, R.C. 1956. Benthic productivity as influenced by fish predation. Limnology and Oceanography. 1:3,162-175.

9. Hunt, B.P., 1953. The life history and economic importance of a burrowing mayfly, *Hexagenia limbata*, in southern Michigan lakes (No. 4). Institute for Fisheries Research, Michigan Department of Conservation cooperating with the University of Michigan.

10. Jerde, C.L., Mahon, A.R., Chadderton, W.L. and Lodge, D.M. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters. 4:2, 150-157.

11. Klump, J. V., Edgington, D. N., Sager, P. E., & Robertson, D. M., 1997. Sedimentary phosphorus cycling and a phosphorus mass balance for the Green Bay (Lake Michigan) ecosystem. Can. J. Fisheries and Aquatic Sciences. 54:1, 10-26.

12. Madenjian, C. P., Schloesser, D. W., & Krieger, K. A., 1998. Population models of burrowing mayfly recolonization in western Lake Erie. Ecological Applications. 8:4, 1206-1212.

13. Neave, F., 1932. A study of the may flies (*Hexagenia*) of Lake Winnipeg. Contributions to Canadian Biology and Fisheries. 7:1, 177-201.

14. O'brien, W.J., Hershey, A.E., Hobbie, J.E., Hullar, M.A., Kipphut, G.W., Miller, M.C., Moller, B. and Vestal, J.R. 1992. Control mechanisms of arctic lake ecosystems: a limnocorral experiment. Hydrobiologia. 240:1-3, 143-188.

15. Sweeney, B.W. and Vannote, R.L. 1987. Geographic parthenogenesis in the stream mayfly Eurylophella funeralis in eastern North America. Holarctic Ecology. 52-59.

16. Thomsen, P., Kielgast, J.O.S., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., Orlando, L. and Willerslev, E. 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular ecology. 21:11, 2565-2573.

17. Tréguier, A., Paillisson, J.M., Dejean, T., Valentini, A., Schlaepfer, M.A. and Roussel, J.M. 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. Journal of Applied Ecology. 51:4, 871-879.

18. Wisconsin Department of Natural Resources (WDNR) (1988). Lower Green Bay Remedial Action Plan for the Lower Green Bay and Fox River Area of Concern. Wisconsin Department of Natural Resources, P.O. Box 7921, Madison, WI 53707.

19. Wisconsin Department of Natural Resources (WDNR) (2009). Lower Green Bay and Fox River Area of Concern Beneficial Use Impairment Delisting Targets. Wisconsin Department of Natural Resources, P.O. Box 7921, Madison, WI 53707.

20. Wisconsin Department of Natural Resources (WDNR) (2013). Stage 2 Remedial Action Plan Update for the Lower Green Bay and Fox River Area of Concern. Wisconsin Department of Natural Resource Office of the Great Lakess, P.O. Box 7921, Madison, WI 53707.

21. Wisconsin Department of Natural Resources (WDNR) (2014). Stage 2 Remedial Action Plan Update for the Lower Green Bay and Fox River Area of Concern. Wisconsin Department of Natural Resource Office of the Great Lakess, P.O. Box 7921, Madison, WI 53707.

CHAPTER 3

Meiobenthos Density and Diversity in the Lower Green Bay and Fox River Area of Concern

Introduction

In 1972, the US-Canada International Joint Commission (IJC) enacted the Basin-wide Great Lakes Water Quality Agreement, and in 1987, 60 'Areas of Concern' (AOC) were established. Remedial Action Plans (RAPs) were later developed in cooperation with state and provincial governments with the goal of identifying specific problems and detailing methods to correct them. The Lower Green Bay and Fox River AOC was one of the originally designated sites, extending from the De Pere Dam on the Fox River to an arbitrary line in lower Green Bay running from Point au Sable on the eastern shore to Longtail Point on the western shore (Fig. 1). As the Green Bay watershed's main river system, the Fox flows through one of the most industrialized and agriculturally developed river valleys in the United States (Klump et al 1997) and contributes as much as 70% of total nutrient loads entering the bay (Dolan and Chapra 2012, Harris and Christie 1987). In response to heavy organic enrichment, a substantial trophic gradient exists with proximity to the Fox River's discharge (Miller and Saylor 1985), where production is fueled to the point of hypereutrophy in much of lower Green Bay, while conditions remain meso- to oligotrphic in the northern bay (Richman et al 1984, Sager and Richman 1990, Sager and Richman 1991). The AOC continues to face challenges associated with remediation of organic pollution and in some cases, potentially with the metrics utilized to measure the degree of such remediation.

The phrase 'Degradation of Benthos' first appeared in the 1993 Remedial Action Plan for the Lower Green Bay and Fox River AOC, compiled by the Wisconsin Department of Natural Resources (WDNR). The five most recent AOC RAPs (2009, 2011, 2012, 2013, and 2014) included 'Beneficial Use Impairment' (BUI) delisting targets, or remediation goals aimed at specific degradation issues within the AOC, including a 'Degraded Benthos' BUI. A principal criterion upon which this BUI can be remediated

(i.e., a suitable delisting target described in the report is met) involves analyzing the composition of benthic macroinvertebrate communities via indices of biological integrity (IBIs). Although valuable in their assessment of habitat quality, they largely overlook the meiobenthic community, which has failed to gain mention in any of the lower Green Bay AOC's Remedial Action Plans.

Meiobenthos can be generally defined as invertebrates which will pass through 1 mm mesh but retained by 45 μm mesh (Higgins and Thiel 1988). Although less commonly used in assessment of benthic habitat in comparison with macroinvertebrates, there have been suggestions for the use of meiobenthos as indicators of habitat quality in organically polluted, nutrient enriched systems (Carriço et al 2013, Kennedy and Jacoby 1999, Moore and Bett 1989). Taxanomic groups such as Copepoda and Ostracoda have been cited specifically (Külköylüoğlu 2004, Külköylüoğlu 2005, Ruiz et al 2004), as negative correlations in their abundances have been observed along organic pollution gradients (Austen et al 1989, Mezquita et al 1999) like that in lower Green Bay. Meiobenthos diversity has also been suggested in some cases to respond negatively to organic pollution and eutrophication (Carriço et al 2013, Gee et al 1985, Heip et al 1988, Moreno et al 2008). This study compares population densities of Copepoda, Ostracoda, and total meiobenthos at five nearshore sampling sites in lower Green Bay—two within and three outside the Lower Green Bay and Fox River AOC boundary. It is important to note that one sample site outside this AOC, in the Menominee River estuary, lies within the boundary of another AOC (Lower Menominee River Area of Concern). The primary rational for listing the Lower Menominee River AOC involved historic arsenic pollution, much of which has since been remediated or is in late stages of remediation (WDNR 2013). The argument could be made that, in terms of organic enrichment, this site more closely resembles my other sample sites outside the Lower Green Bay and Fox River AOC compared to those within, and thus can be adequately used for comparison to Lower Green Bay and Fox River AOC sites in this study. For example, the US Geological Survey reported total phosphorus (limiting nutrient in Green Bay, Klump et al. 1997) concentrations at a site in close proximity to my Menominee River estuary site to average 27 $\mu g\ L^{-1}$ in 2014 (Water Quality Samples for Wisconsin: Sample Data,

2014, http://nwis.waterdata.usgs.gov/wi/nwis/qwdata?). This value more closely reflects the average of total phosphorus concentrations from the same USGS report for a similar site near the Oconto River estuary (35 μg L^{-1}) (2009 data was the most recent available for this site) as well as for a site in close proximity to my other non-AOC site (40 μg L^{-1}, reported by NEW water, formerly the Green Bay Metropolitan Sewerage District, in 2015), as opposed to total phosphorus concentrations reported near my sites within the Lower Green Bay and Fox River AOC, which averaged more than double these concentrations.

Taxon (order) richness and evenness, as well as diversity (Shannon-Wiener and Simpson's heterogeneity indices) were also calculated for all meiobenthos collected and compared between sites within and outside the Lower Green Bay and Fox River AOC. Overall meiobenthos abundance and diversity were expected to be lower at sites within the AOC. Shannon-Wiener diversity gives more weight to 'rare' or less common taxa (Type I index), while numerically dominant species are more heavily represented by Simpson's index of diversity (Type II index). Calculating their ratio was thus aimed at illustrating the relative influence of rare taxa at a given site (i.e. a higher ratio means rare taxa were more proportionally important). The ratio of Shannon-Wiener diversity to Simpson's index was expected to be greater for sites outside the AOC, as more rare taxa were expected to be present with a lesser degree of organic enrichment. Sediment water content and grain size fractions were also calculated for each sample site.

Methods

Meiobenthos collection, water content, and grain size fractions

Six petite (6x6 inch; ~15x15 cm) Ponar grab samples were taken at each of five sites on June 29, 2015, "Cat Islands", "Longtail Point", "Littletail Point", "Oconto River estuary", and "Menominee River estuary" (Fig. 4). Cat Islands and Longtail Point sites reside within the boundaries of the Lower Green

Figure 4. Meiobenthos sampling sites in lower Green Bay. Site 1: "Cat Islands" (44° 33.144'N, 88° 1.269'W), site 2: "Longtail Point" (44° 36.902'N, 88° 0.425'W), site 3: "Littletail Point" (44° 40.049'N, 87° 59.390'W), site 4: "Oconto River estuary" (44° 53.526'N, 87° 49.983'W), site 5: "Menominee River estuary" (45° 5.820'N, 87° 36.391'W). Dotted line and enclosed shaded region shows the approximate area encompassed by the Lower Green Bay and Fox River Area of Concern.

Bay and Fox River AOC, while the others are outside. Each grab sample was released into a water-tight metal pan. In addition to more solid sediment collected by the Ponar grab, a significant amount of sediment-laden overlying liquid was present. For each Ponar grab sample taken, this liquid was decanted into separate sample containers. These 'supernatant' samples contained the vast majority of meiobenthos present, and were thus subsampled and used to estimate meiobenthos density and diversity in this study. All samples were stored in a portable cooler until subsampling in the lab.

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 ± standard deviations (n = 3 for each site).

Subsampling procedure and population density, richness, evenness, and diversity calculation

Prior to inspection, the volume contained in each supernatant 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 in an approximately even distribution. Subsamples were inspected under a 10x dissecting microscope and meiobenthos 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, and that the same probability of encountering a given number of fauna existed for each subsample.

Meiobenthos encountered in each subsample were identified to order level. To estimate ostracod, copepod, and total meiobenthos abundance in a petite Ponar grab sample, the number of individuals in a given 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 (area in square meters sampled by one petite Ponar grab) to give density m^{-2} values (reported as means ± standard errors). Statistical significance comparisons between ostracod, copepod, and total meiobenthos densities at sites within the Lower Green Bay and Fox River AOC versus sites outside the AOC were calculated using a two-tailed Mann-Whitney U-test.

Taxon (order) richness, evenness, Shannon-Wiener diversity and Simpson's index of diversity (1-D) were calculated for all meiobenthos collected in each sample (n = 6 per site). Sample values were then averaged to obtain richness, evenness, and diversity values for each study site. Richness (R) refers to the number of orders present at a given site. Shannon-Wiener diversity (H') (Shannon and Weaver 1949) is described by Equation 1, evenness (E) is described by Equation 2, and Simpson's index of diversity ($1 - D$) (Simpson 1949) is described by Equation 3.

(1) $H' = -\sum_{i=1}^{O} p_i \ln p_i$

(Where O is the summed population density of all orders present, and p_i is the proportion of O made up of the ith order)

(2) $E = H' / \ln R$

(3) $(1 - D) = 1 - [\sum n(n-1) / N(N\text{-}1)]$

(Where (n) is the sum of the products of each order's population density in a sample, and (N) is the population density of all meiobenthos in the sample)

The ratio of Shannon-Wiener diversity to Simpson's index of diversity ($H' / 1 - D$) was also calculated for all sites. Two-tailed Mann-Whitney U-tests were used for comparisons of statistical significance in regard to richness, evenness, and diversity between sites. Population densities and diversity-based statistics are reported as averages ± standard deviations.

Results

A total of 277 meiobenthos were collected from subsamples, including Ostracoda, Copepoda (harpacticoid, calenoid, and cyclopoid forms present with harpacticoids dominating), Nematoda (Chromadorea), Arachnida (Hydrachnidae), and Branchiopoda (Chydoridae). Ostracods and Copepods accounted for the majority, with 124 and 81 individuals collected, respectively. Average water content ranged from 36.0% (Menominee River estuary) to 55.6% (Cat Islands), sand content ranged from 80.7% (Menominee River estuary) to 75.9% (Cat Islands), silt content ranged from 12.7% (Menominee River estuary) to 21.3% (Cat Islands), and clay content ranged from 0.0% (Menominee River estuary) to 5.0% (Longtail Point) (Table 6).

Average ostracod density m^{-2} was calculated to be 8714 ± 1392 at Cat Islands, 12,479 ± 1645 at Longtail Point, 17,065 ± 3199 at Littletail Point, 5903 ± 1152 at the Oconto River estuary, and 10,272 ± 1340 at the Menominee River estuary (Fig. 5). Copepod density m^{-2} averaged 7159 ± 584 at Cat Islands, 14,221 ± 3457 at Longtail Point, 6482 ± 3006 at Littletail Point, 3524 ± 2475 at the Oconto River estuary, and 4158 ± 589 at the Menominee River estuary (Fig. 6). Total meiobenthos density m^{-2} averaged 20,483 ± 2805 at Cat Islands, 32,835 ± 3496 at Longtail Point, 30,909 ± 7132 at Littletail Point, 10,338 ± 2028 at the Oconto River estuary, and 21,839 ± 2180 at the Menominee River estuary (Fig. 7). Ostracod density was significantly greater at Longtail Point than at the Oconto River estuary ($p = 0.031$) (Fig. 5). 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 ($p = 0.013$) (Fig. 6). Total meiobenthos density was also significantly greater at Cat Islands than at the Oconto River estuary ($p = 0.025$) and was greater at Longtail Point than at the Oconto River estuary ($p = 0.005$) and at the Menominee River estuary ($p = 0.031$) (Fig. 7).

		%water	%Sand	%Silt	%Clay	Porosity
Cat Islands						
	$\bar{x}$	55.6	75.9	21.3	2.9	1.02
	SD	6.2	4.4	5.1	0.8	0.14
Longtail Point						
	$\bar{x}$	40.8	81.8	13.2	5.0	1.36
	SD	2.1	2.7	2.9	1.4	0.05
Littletail Point						
	$\bar{x}$	39.6	76.3	19.5	4.2	1.39
	SD	3.8	1.0	1.4	1.3	0.09
Oconto R. estuary						
	$\bar{x}$	45.8	80.5	19.1	0.4	1.25
	SD	22.5	29.0	29.0	0.1	0.52
Menominee R. estuary						
	$\bar{x}$	36.0	87.3	12.7	0.0	1.47
	SD	13.9	14.5	14.5	0.0	0.32
AOC Ave. ± SD		***48.2 ± 10.4***	***78.8 ± 4.2***	***17.2 ± 5.7***	***3.9 ± 1.5***	***1.19 ± 0.24***
non-AOC Ave. ± SD		***40.5 ± 4.9***	***81.3 ± 5.5***	***17.1 ± 3.8***	***1.5 ± 2.3***	***1.37 ± 0.11***
Ave. ± SD (all sites)		***43.6 ± 7.6***	***80.3 ± 4.7***	***17.1 ± 3.9***	***2.5 ± 2.2***	***1.30 ± 0.17***

Table 6. Sample averages and standard deviations (n=3) for water content, grain size fractions, and porosity at lower Green Bay sample sites. Averages and standard deviations (Ave. ± SD) also calculated for AOC sites (n=2), non-AOC sites (n=3), and all sites sampled (n=5).

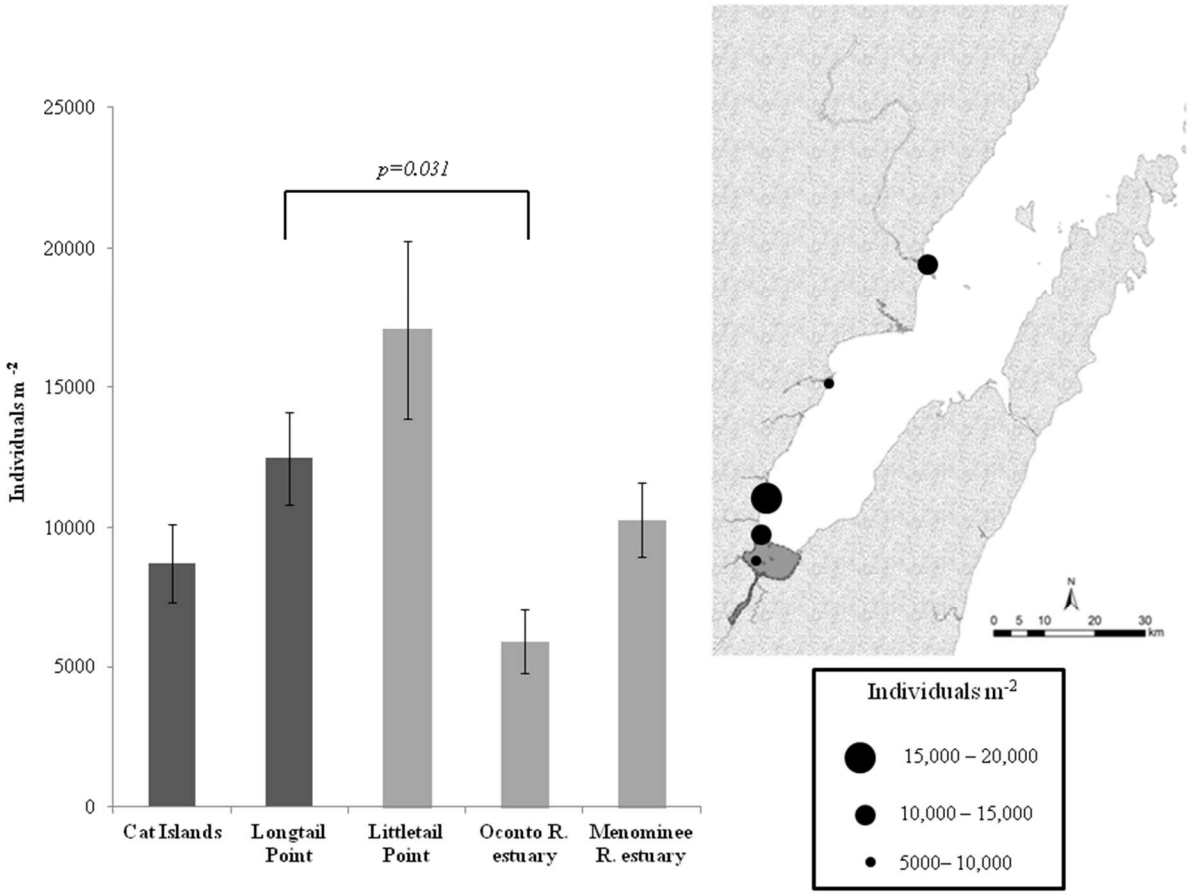

Figure 5. Ostracod population densities**.** Error bars represent standard error of the mean. Darker bars represent sites within the Lower Green Bay and Fox River Area of Concern. P-values denote significant differences ($p < 0.05$) in population densities between samples obtained at the two sites at the ends of each bar.

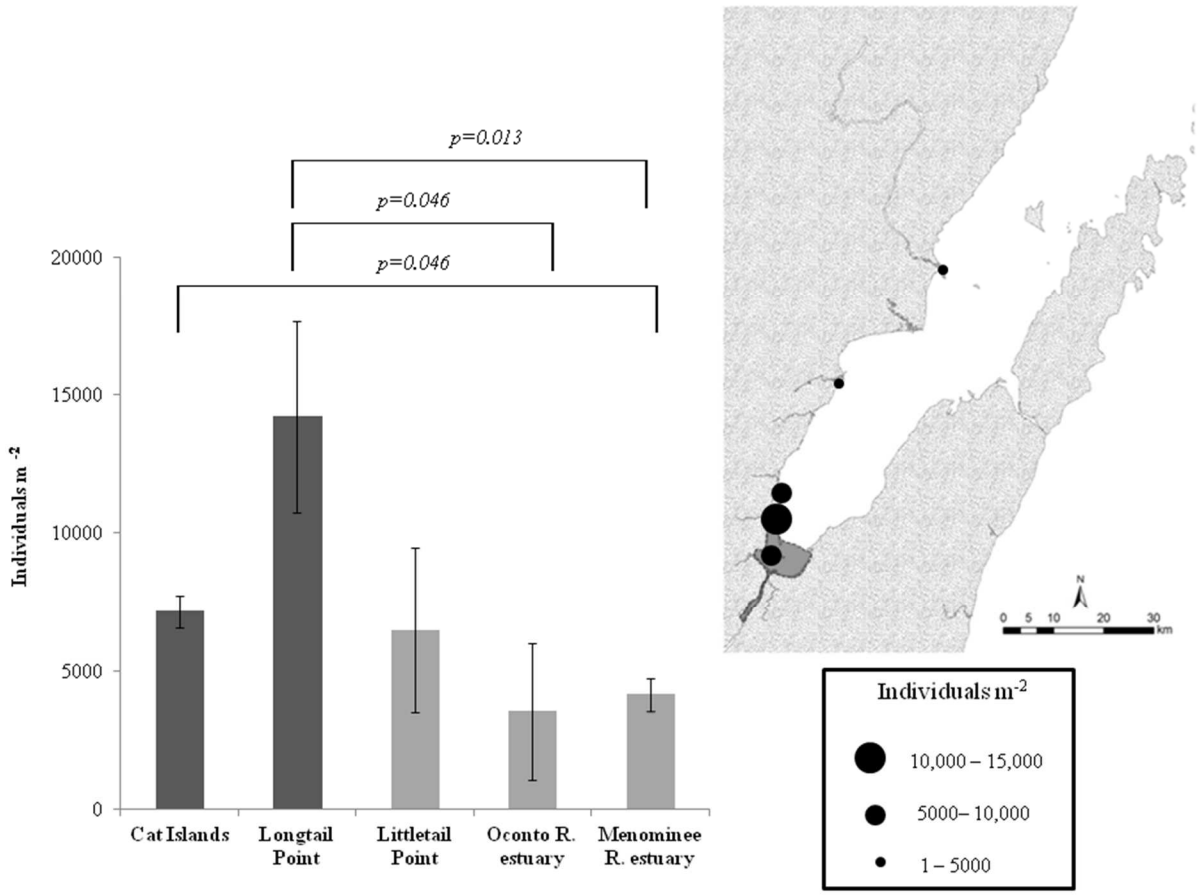

Figure 6. Copepod population densities. Error bars represent standard error of the mean. Darker bars represent sites within the Lower Green Bay and Fox River Area of Concern. P-values denote significant differences ($p < 0.05$) in population densities between samples obtained at the two sites at the ends of each bar.

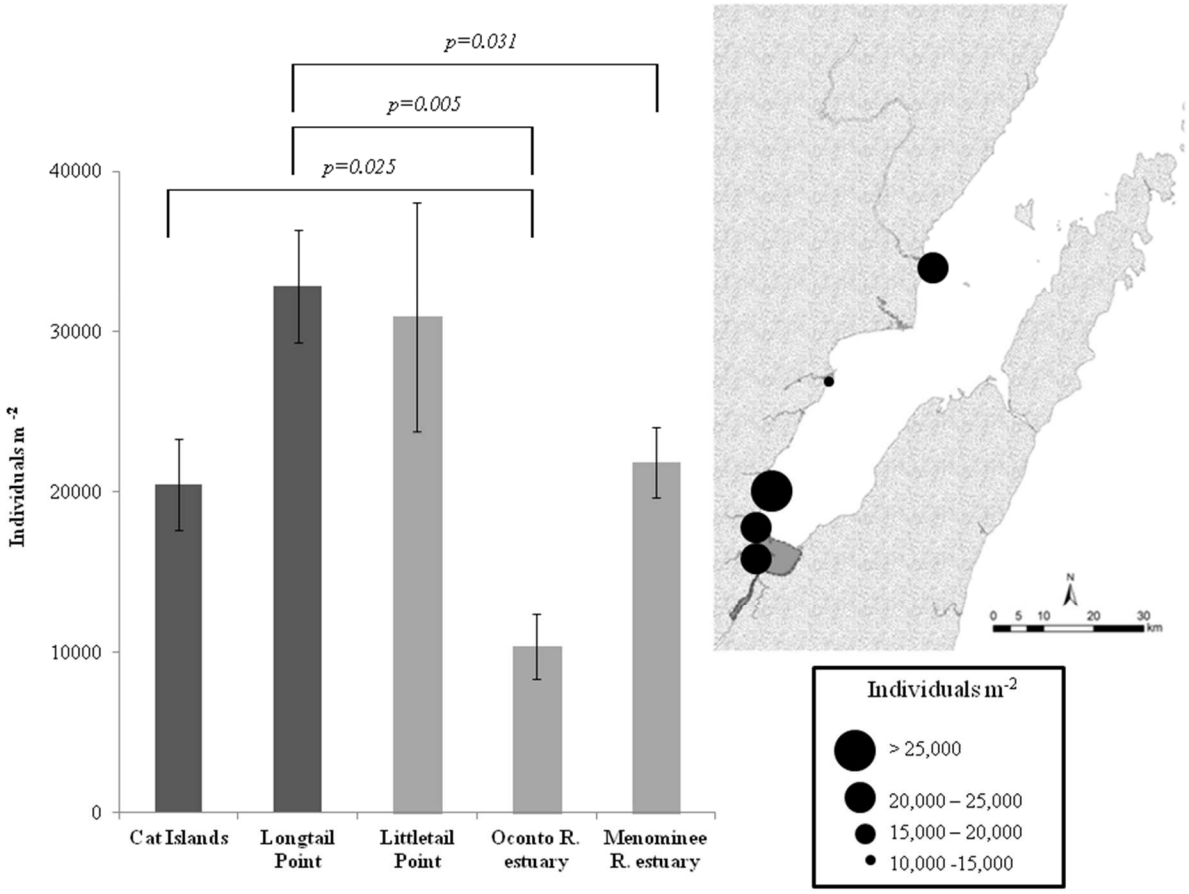

Figure 7. Total meiobenthos population densities. Error bars represent standard error of the mean. Darker bars represent sites within the Lower Green Bay and Fox River Area of Concern. P-values denote significant differences ($p < 0.05$) in population densities between samples obtained at the two sites at the ends of each bar.

		Richness (R)	Evenness (E)	Shannon-Wiener diversity (H')	Simpson's index of diversity (1- D)	H' / 1- D
Cat Islands						
	$\bar{x}$	3.17	0.92	1.03	0.62	1.67
	SD	0.75	0.07	0.22	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 7. Sample averages and standard deviations (n=6) for taxon (order) richness, evenness, Shannon-Wiener 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.

Taxon (order) richness, evenness, Shannon-Wiener diversity, Simpson's index of diversity, and $H'/\ 1-D$ for all sites are given in Table 7. 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-Wiener 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 ($p = 0.008$).

Discussion

Of the sites sampled in this study, no site outside the Lower Green Bay and Fox River AOC boundary had a significantly higher population density of Ostracoda, Copepoda, or total meiobenthos than a site within the AOC. Conversely, densities were in several cases higher within the AOC. If the assumption is true that meiobenthos densities respond negatively to organic enrichment, benthic habitat quality within the AOC, at least at the western nearshore sites sampled for this study, may be higher than expected. Richness, evenness, diversity, and $H'/\ 1-D$ were not significantly greater at any site outside the Lower Green Bay and Fox River AOC site compared to sites within. These parameters were relatively consistent between sites with the exception of the Oconto River estuary (richness and diversity were significantly lower compared to both sites within the AOC, and $H'/\ 1-D$ was significantly lower compared to Longtail Point). If benthic habitat quality as impacted by organic pollution was lower at western nearshore sites within the AOC than at sites outside, my results did not provide evidence for this claim in regard to meiobenthos abundance or diversity.

Average sediment water content and grain size fractions were relatively consistent between sites (Table 1). One of the three samples taken at the Oconto River estuary was notably lower in sand content

and higher in silt content (as is reflected in the high standard deviation values reported in Table 1). The other two samples from the same site (average ± standard deviation of 97.2 ± 2.6% sand, 2.3 ± 2.5% silt, 0.5 ± 0.1% clay) align more closely in regard to grain size fractions to samples from other sites, and also align closely with grain size data from a site in close proximity to our Oconto River estuary site sampled by the US Geological Survey in 2012 (average ± standard deviation of 97.3 ± 1.5% sand, 2.0 ± 1.7% silt, 0.7 ± 0.6% clay, n = 3) (Eikenberry et al 2014). That said, the fact that we obtained a sample of notably different grain size fractions (i.e. much siltier) may suggest a patchy distribution of sediment type in regard to grain size in the Oconto River estuary. Whether or not the potentially patchy sediment distribution at this site influenced the comparative lack in meiobenthos abundance or diversity here versus at other sample sites remains unknown. Some ostracod species may prefer sandier substrates (Benzie 1989), but more literature points to a lack of significance in the correlation between sediment grain size fractions alone and ostracod distribution (Szlauer-Lukaszewska 2015, Külköylüoğlu 2000). The same appears to be true in regard to copepods (Hockin 1983, Ravenel and Thistle 1981) and for meiobenthos in general (de Bovée et al 1989). Sediment preferences of meiobenthic taxa may be more dependent on differences in microbes attached to sediment particles (de Bovée et al 1989, Ravenel and Thistle 1981), food availability based on surface areas of different sized sediment particles (Marcotte 1986), temperature and dissolved oxygen (Yilmaz and Külköylüoğlu 2006), or "other hydrographic or biological factors" (Ravenel and Thistle 1981) rather than on grain size itself.

It is worth noting that there were no significant differences in ostracod, copepod, or total meiobenthos abundance or meiobenthos diversity, richness, or evenness between Longtail Point and Littletail Point. These two sample sites were nearly identical in depth and both are located beneath similar southward facing peninsulas, which made them close to ideal for comparisons between conditions within and outside the AOC. They differ substantially however in terms of nutrient enrichment, as outputs from the Fox River appear to have a much greater influence at Longtail Point. Two sites sampled in close proximity to Longtail Point and Littletail Point by NEW Water yielded average total phosphorous

concentrations near Longtail Point approximately double to those near Littletail Point (85 μg L^{-1} and 40 μg L^{-1} respectively) in summer 2015. Based on our observations while sampling in June 2015, phytoplankton was dense enough to limit visibility to about 30 cm at Longtail Point (the same was the case at Cat Islands), while at Littletail Point no phytoplankton was observable and the bottom was clearly visible from the boat. Live and detrital phytoplankton (i.e. diatoms, microalgae), present in much higher quantities due to nutrient enrichment at Longtail Point, represent major food sources for meiofauna (Fenchel 1978, Franco et al 2008, Joint et al 1982, Mann 1988) and may have played a role in their comparable densities and diversity to those encountered at Littletail Point.

Although several studies point to a negative correlation between the degree of organic enrichment and meiobenthos density or diversity (as mentioned in the introduction), a few report the opposite correlation in some cases. Gee et al (1985) reported harpacticoid copepod density increased significantly in mesocosms treated with high doses of powdered brown algae (*Ascophyllum nodosum*) compared to controls. Ristau et al (2012) reported increases in ostracod density in mesocosa treated with high doses of phosphorus (up to 250 μg L^{-1} total phosphorus concentration). Other taxa (e.g., Copepoda, Nematoda) saw declines at these doses however, and it might be arguable that the ostracod density increase was more attributable to a comparative lack of competition for food with fewer other meiobenthos present. The same study also concluded that total meiobenthos density peaked at a total phosphorus concentration of 30 μg L^{-1}, close to the concentration reported by NEW Water in proximity to our sites outside the Lower Green Bay and Fox River AOC. Still, other studies concluded that more research was needed to accurately understand the relationship between meiobenthos density or diversity and organic enrichment. Hockin (1983) stated "*more basic research is needed to assess response of a variety of meiobenthic communities to various loads of organic pollution*". Mitwally and Fleeger (2012) suggest that "*natural variability was greater than variability induced by fertilization* [organic enrichment]", and that "*a better mechanistic understanding of the relationship between benthic macroalgae and meiofaunal abundance is needed to fully understand how nutrient enrichment affects meiofauna*". Based on these conclusions and

the varying results of other studies, it is not yet entirely clear whether or not the meiobenthic community can be reliably used to assess habitat quality in lower Green Bay due to its response(s) to organic enrichment. That said, both the similarities and differences in meiobenthos density and diversity between sites within and outside the Lower Green Bay and Fox River AOC shown by our results may still provide information regarding other components of habitat quality (e.g., food availability and its relationship to trophic dynamics within and outside the AOC), and may serve as a starting point for future research of meiobenthic community dynamics in lower Green Bay. Continued sampling of meiobenthos at other sites (including offshore) within and outside the AOC and possibly more importantly, at multiple times throughout a given year to examine the effects of seasonality on meiobenthos abundance and community structure, will be necessary in such research. If it can be determined that meiobenthos-based metrics can be reliably used in benthic habitat quality assessment in regard to organic enrichment, it may be worth considering whether data from such metrics could be added to the delisting criteria of the Degraded Benthos BUI, and/or that the currently arbitrary AOC boundary might be adjusted to account for higher habitat quality, as measured by meiobenthic community dynamics in certain regions.

References

1. Austen, M.C., Warwick, R.M. and Rosado, M.C., 1989. Meiobenthic and macrobenthic community structure along a putative pollution gradient in southern Portugal. Marine Pollut. Bull. 20:8, 398-405.

2. Benzie, J.A. 1989. The distribution and habitat preference of ostracods (Crustacea: Ostracoda) in a coastal sand-dune lake, Loch of Strathbeg, north-east Scotland. Freshwater Biology. 22:2, 309-321.

3. Carriço, R., Zeppilli, D., Quillien, N. and Grall, J., 2013. Can meiofauna be a good biological indicator of the impacts of eutrophication caused by green macroalgal blooms. Les cahiers nat. de l'Observatoire marin. 2:1, 9-16.

4. de Bovée, F., Guidi, L.D. and Soyer, J., 1990. Quantitative distribution of deep-sea meiobenthos in the northwestern Mediterranean (Gulf of Lions). Continental Shelf Res. 109, 1123-1145.

5. Dolan, D.M. and Chapra, S.C. 2012. Great Lakes total phosphorus revisited: 1. Loading analysis and update (1994–2008). J. Great Lakes Res. 38:4, 730-740.

6. Eikenberry, B. C. S., Bell, A. H., Burns, D. J., & Templar, H. A., 2014. Benthos and plankton community data for selected rivers and harbors along Wisconsin's Lake Michigan shoreline, 2012 (No. 824). US Geological Survey.

7. Fenchel, T.M., 1978. The ecology of micro-and meiobenthos. Annu. Rev. Ecology and Systematics. 9, 99-121.

8. Franco, M.A., Soetaert, K., Van Oevelen, D., Van Gansbeke, D., Costa, M.J., Vincx, M. and Vanaverbeke, J., 2008. Density, vertical distribution and trophic responses of metazoan meiobenthos to phytoplankton deposition in contrasting sediment types. Marine Ecology Prog. Ser. 358, 51.

9. Gee, J.M., Warwick, R.M., Schaanning, M., Berge, J.A. and Ambrose, W.G., 1985. Effects of organic enrichment on meiofaunal abundance and community structure in sublittoral soft sediments. Journal of Experimental Marine Biology and Ecology. 91:3, 247-262.

10. Harris, V.A., and Christie, J. 1987. The lower Green Bay Remedial Action Plan: nutrient and eutrophication management. Technical Advisory Committee report. Publication No. WR-167-87. Wisconsin Department of Natural Resources, Madison, Wis. *In*: Klump, J. V., Edgington, D. N., Sager, P. E., & Robertson, D. M., 1997. Sedimentary phosphorus cycling and a phosphorus mass balance for the Green Bay (Lake Michigan) ecosystem. Can. J. Fisheries and Aquatic Sciences. 54:1, 10-26.

11. Heip, C., Warwick, R.M., Carr, M.R., Herman, P.M., Huys, R., Smol, N. and Van Holsbeke, K., 1988. Analysis of community attributes of the benthic meiofauna of Frierfjord/Langesundfjord. Marine Ecology Prog. Ser. 46, 171-180.

12. Heip, C. 1995. Eutrophication and Zoobenthos dynamics. Ophelia. 41:1, 113-136.

13. Higgins, R.P. and Thiel, H., 1988. Introduction to the Study of Meiofauna. Smithsonian Institution Press, Washington D.C.

14. Hockin, D.C., 1983. The effects of organic enrichment upon a community of meiobenthic harpacticoid copepods. Marine Environ. Res. 10:1, 45-58.

15. Joint, I.R., Gee, J.M. and Warwick, R.M., 1982. Determination of fine-scale vertical distribution of microbes and meiofauna in an intertidal sediment. Marine Biology. 72:2 , 157-164.

16. Kennedy, A.D. and Jacoby, C.A., 1999. Biological indicators of marine environmental health: meiofauna–a neglected benthic component?. Environ. Monitoring and Assess. 54:1, 47-68.

17. Klump, J. V., Edgington, D. N., Sager, P. E., & Robertson, D. M., 1997. Sedimentary phosphorus cycling and a phosphorus mass balance for the Green Bay (Lake Michigan) ecosystem. Can. J. Fisheries and Aquatic Sciences. 54:1, 10-26.

18. Külköylüoğlu, O., 2000, September. The importance of cosmopolitan and indicator species of Ostracoda (Crustacea) in Turkey based on some water parameters. In Sinop Water Product Conference. 2000, 421-437.

19. Külköylüoğlu, O. 2004. On the usage of ostracods (Crustacea) as bioindicator species in different aquatic habitats in the Bolu region, Turkey. Ecological Indicators. 4:2, 139-147.

20. Külköylüoğlu, O. 2005. Factors affecting the occurrence of ostracoda (crustacea) in the Yumrukaya reedbeds (Bolu, Turkey). Wetlands. 25:1, 224-227.

21. Mann, K.H., 1988. Production and use of detritus in various freshwater, estuarine, and coastal rnarine ecosystems. Limnol. and Oceanogr. 33, 910-930.

22. Marcotte, B.M., 1986. Sedimentary particle sizes and the ecological grain of food resources for meiobenthic copepods. Estuarine, Coast. and Shelf Science. 23:3, 423-427.

23. Mezquita, F., Hernández, R. and Rueda, J. 1999. Ecology and distribution of ostracods in a polluted Mediterranean river. Palaeogeogr., Palaeoclimatology., Palaeoecology. 148:1, 87-103.

24. Miller G.S. and Saylor G. H. 1985. Currents and temperatures in Green Bay, Lake Michigan. J. Gt. Lakes Res. 11: 97–109.

25. Mitwally, H.M. and Fleeger, J.W., 2013. Long-term nutrient enrichment elicits a weak density response by saltmarsh meiofauna. Hydrobiologia. 713:1, 97-114.

26. Moore, C.G. and Bett, B.J. 1989. The use of meiofauna in marine pollution impact assessment*. Zoological J. Linnean Soc. 96:3, 263-280.

27. Moreno, M., Vezzulli, L., Marin, V., Laconi, P., Albertelli, G. and Fabiano, M., 2008. The use of meiofauna diversity as an indicator of pollution in harbours. ICES J. Marine Science: Journal du Conseil. 65:8, 1428-1435.

28. Ravenel, W.S. and Thistle, D., 1981. The effect of sediment characteristics on the distribution of two subtidal harpacticoid copepod species. J. Exp. Marine Biology and Ecology. 50:2, 289-301.

29. Richman, S., Sager, P. E., Banta, G., Harvey, T. R., & DeStasio, B. T. (1984). Phytoplankton standing stock, size distribution, species composition and productivity along a trophic gradient in Green Bay, Lake Michigan. Verhandlung Internationale Vereinigung Limnologie. 22:1.

30. Ristau, K., Faupel, M. and Traunspurger, W., 2012. The effects of nutrient enrichment on a freshwater meiofaunal assemblage. Freshwater Biology. 57:4, 824-834.

31. Ruiz, F., González-Regalado, M.L., Borrego, J., Abad, M. and Pendón, J.G., 2004. Ostracoda and foraminifera as short-term tracers of environmental changes in very polluted areas: the Odiel Estuary (SW Spain). Environ. Pollut. 129:1, 49-61.

32. Sager, P. E., & Richman, S. (1990). Patterns of Phytoplankton-Zooplankton Interaction Along a Trophic Gradient: I. Production and Utilization. Internationale Vereinigung fuer Theoretische und Angewandte Limnologie. Verhandlungen IVTLAP. 24:1.

33. Sager, P. E., & Richman, S. (1991). Functional interaction of phytoplankton and zooplankton along the trophic gradient in Green Bay, Lake Michigan. Can. J. Fisheries and Aquatic Sciences. 48:1, 116-122.

34. Shannon, C.E. and Weaver, W. 1949. The mathematical theory of communication. Urbana, IL.

35. Simpson, E.H. 1949. Measurement of diversity. *Nature*. 163, 688.

36. Szlauer-Lukaszewska, A., Substrate type as a factor affecting the ostracod assemblages in groyne fields of the Oder River (Poland). Northwestern J. Zoology. 11:2, 274-287.

37. Wisconsin Department of Natural Resources (WDNR) (2013). 2013 Remedial Action Plan Update for the Lower Menominee River Area of Concern. Wisconsin Department of Natural Resource Office of the Great Lakes, P.O. Box 7921, Madison, WI 53707.

38. Yılmaz, F. and Külköylüoğlu, O., 2006. Tolerance, optimum ranges, and ecological requirements of freshwater Ostracoda (Crustacea) in Lake Aladağ (Bolu, Turkey). Ecological Res. 21:2, 165-173.