PENNSYLVANIA SEA GRANT

FINAL REPORT REQUIREMENTS

2016

1. **Cover Page**

    a. **Title**: *Investigating the role of contaminants and parasite prevalence in the observed mortality of smallmouth bass in the Susquehanna River Basin*

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    d. **Dates:** November 17, 2014 – July 31, 2016

2. Executive Summary

Since the mid-2000s, young of the year smallmouth bass (*Micropterus dolomieu)* have been documented to have disease characteristics as well as mortality events within the Susquehanna River Basin. In addition to young of the year mortality, documented declines in adult smallmouth bass coupled with the presence of disease characteristics and endocrine disruption further increased concern for the overall health of both juvenile and adult smallmouth bass in the Susquehanna River Basin. A wide range of environmental risk factors are being studied for the role they may play in mediating disease and mortality events in juvenile smallmouth bass, including the presence of parasites and contaminants in the environment. Through collaborative efforts, this project aimed to investigate a myxozoan parasite infecting young of the year smallmouth bass and contaminants present in part of Susquehanna River Basin. Although the myxozoan parasite has been previously identified, little else is known regarding its life history and invertebrate host that it may use (an aquatic oligochaete). In addition, little information is currently available on the chemical constituents that are present in young of the year tissues. During 2014 and 2015, aquatic oligochaete samples as well as fish contaminant samples were collected from the West Branch of the Susquehanna River Basin. Myxozoan DNA was found in a subset of aquatic oligochaete samples (11 out of 506) collected in 2014, but none matched the target myxozoan (*Myxobolus inornatus)*. Also in 2014, samples were collected from five sample sites in the West Branch of the Susquehanna River to determine the presence of a variety of chemical compounds in 34 young of the smallmouth bass. A site located on the West Branch of the Susquehanna River had the greatest number of contaminants detected. Samples collected for contaminant analysis collected in 2015 have not yet been analyzed (the samples have been sent to the lab, but results have not been received). Although the sample size and spatial extent of sampling was somewhat limited, 2014 data did provide insight on the worm species that are present in the West Branch of the Susquehanna River basin that may play a role in the myxozoan parasite life cycle, as well as providing the first information on the contaminants that are present in young of the year smallmouth bass. Future research can build upon current findings to continue to investigate the role both parasites and contaminants may play in the onset of disease and mortality in juvenile smallmouth bass.

3.) Report

a.) Introduction

Smallmouth bass are a socioeconomically and ecologically important species, supporting important recreational fisheries throughout the United States, including in Pennsylvania. During the late 1990s and 2000s, however, their populations have been in decline in several major rivers including the

Susquehanna River and its tributaries. For example, there has been a decrease in total catch per effort of smallmouth bass in Susquehanna River from the 1990s to the early 2000s, and a more recent (i.e., the past 10 years) decline in the West Branch Susquehanna River. In fact there is a > 95% probability of annual declines occurring between the late 1990s and early 2000s for the Susquehanna River and a 70 – 75% probability of annual declines from the early 2000s through 2011 in the West Branch Susquehanna River (PA Fish and Boat Commission annual survey data, T. Wagner, U.S. Geological Survey, unpublished analysis). In addition to adult declines, disease and mortality of young of the year (YOY) were documented starting in 2005 in the West Branch of the Susquehanna, Susquehanna, and Juniata rivers (Smith et al. 2015).

   The causes of the decline of smallmouth bass populations observed in some Pennsylvania rivers are unclear. However, Allen et al. (2008) reviewed annual exploitation and total mortality estimates for populations of largemouth bass *M. salmoides* and found that fishing mortality rates and annual exploitation rates have declined since the 1990s. Allen et al. (2008) suggested that the observed trends were likely due to an increase in the voluntary release of fish by anglers. It is reasonable to assume that this trend of increasing voluntary release has also occurred for many riverine smallmouth bass populations, including those in Pennsylvania, and that the declines observed in Pennsylvania are not due to an increase in harvest by anglers. An alternative hypothesis is that the declines are related to environmental stressors (e.g., pesticides, endocrine-modulating substances) and pathogens that directly or indirectly increase mortality. A variety of bacterial pathogens, viruses and parasites have been isolated in Pennsylvania smallmouth bass populations (both juveniles and adults; V. Blazer, USGS, personal communication) and other large riverine systems, including the Potomac River (Blazer et al. 2010). Although no one pathogen has been identified as the causative agent of mortality, results of previous work suggests that a variety of stressor may result in compromised immune systems, whereby smallmouth bass succumb to a variety of opportunistic pathogens (Blazer et al. 2010). Blazer et al. (2011) also found positive correlations between testicular oocytes (intersex) in male smallmouth bass within the Potomac River drainage and the percentage of agricultural land use within the catchment, total number of animal feeding operations, and the number of poultry houses. Intersex has also been documented in smallmouth bass within the Susquehanna River Basin where again a correlation with agricultural land use was found, in addition to increases in prevalence and severity when compared to other river drainages in Pennsylvania (e.g., Allegheny and Ohio River) (Blazer et al. 2014). Thus, catchment land use could have implications for the overall condition of riverine smallmouth bass populations, including those in Pennsylvania.

In response to the observed declines in abundance of smallmouth bass in the Susquehanna River basin, considerable state and federal resources have been devoted to examining potential disease-causing agents, environmental conditions, and contaminants that may be contributing to mortality in adult and young-of-year smallmouth bass. Through a collaborative effort by the PA Fish and Boat Commission, PA Department of Environmental Protection, U.S. Geological Survey, and the PA Cooperative Fish and Wildlife Research Unit at Penn State University a wide range of physical and biological in-river and landscape characteristics are being studied that are hypothesized to be contributing to mortality. Two risk factors that may be playing a role in the observed disease and mortality in smallmouth bass are contaminants and myxozoan parasite infection. Contaminants, including endocrine disruptors, metals, organochlorines, and pesticides, not only have direct effects on smallmouth bass, but may play a role in mediating disease, including viruses, bacteria, and parasites. Contaminant analysis of tissue and bed sediment in the Susquehanna River basin will allow investigation of the potential sources of contaminants (i.e., agricultural, industry, etc.) and also characterize compounds of concern (e.g., endocrine disruptors, pesticides; Alvarez et al. 2009). In addition to contaminants, a myxozoan species (*Myxobolus inornatus*) has been determined to be infecting YOY in the Susquehanna River basin (Walsh et al. 2012). Preliminary work by our research team has found myxozoan DNA in worms, but the conclusive link between intermediate host and parasite along with information regarding temporal aspects of parasite release from the intermediate host are still limiting factors in our ability to determine its role in mortality and to manage the system. Identifying the intermediate host(s) of this parasite is a critical component to elucidating the role this parasite plays in mortality events and the environmental conditions that contribute to exposure to this parasite. Thus, additional work is needed to understand the role that contaminants and parasite infection may be playing in disease and mortality and how chemical concentrations and parasite load vary throughout critical periods for smallmouth bass development. The study area for this research also overlapped with areas of previous research including where an adult radiotelemetry study occurred. This will allow a deeper understanding of multiple variables that are being studied including movement dynamics and how they may relate to other stressors (i.e., transport of contaminants). **Therefore, the objectives of this study are to (1) identify the intermediate host(s) for various myxozoan parasites that are potential threats to YOY and adult smallmouth bass, (2) investigate the role and relationships of contaminants in river sediment, adult female smallmouth bass gonads, and YOY tissue as it relates to risk factors for mortality, and (3) link contaminant data with ongoing telemetry work (West Branch of the Susquehanna River Basin) to understand risks for exposure, and.** In this context, the proposed

project will substantially build upon our ongoing multi-agency, multi-university collaborative research effort where existing data on the biological condition of smallmouth bass (e.g., external lesions, microscopic pathology, plasma analyses), fish movement, and thermal habitat conditions have been (and are currently being) collected.

b.) Methodology

Myxozoan Parasite Research 2014

Collection of aquatic oligochaetes

During 2014, aquatic oligochaetes were collected from five sites within the West Branch of the Susquehanna River basin including the telemetry research area (Bald Eagle Creek and Pine Creek). Aquatic oligochaetes were targeted due to their role as an invertebrate host for freshwater myxozoans belonging to the genus *Myxobolus* (Fiala et al. 2015). Other tributary sites were also targeted where ongoing research efforts occurred (i.e., Chillisquaque Creek and
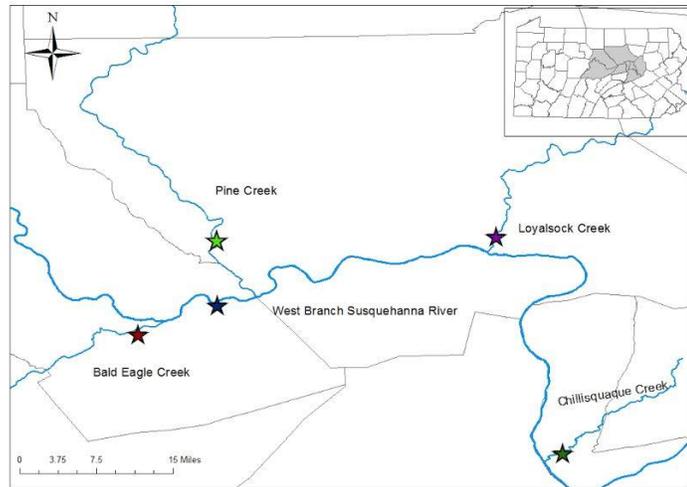

Figure 1: Layout of 2014 aquatic oligochaete sampling sites in the West Branch of the Susquehanna River Basin including four tributary sites and one main stem site (stars). Sampling occurred in the central part of the state (see inset).

Loyalsock Creek) (Figure 1).   These sites have had myxozoan infection documented YOY smallmouth bass during previous sampling years. Aquatic oligochaetes were collected by identifying preferred substrate of aquatic oligochaetes (e.g., decaying leaf litter, sediment) and shoveling substrate into a five gallon bucket. The bucket was filled approximately 1/3 of the way with material and water. Samples were collected between May 2014 and July 2014, encompassing the time period prior to and during the time YOY smallmouth bass were noticed to have parasite infection. After collection, samples were sifted using multiple sieves stacked with the finest mesh size of 60. Aquatic oligochaetes were picked from the sample and each individual was placed in a vial of 95% non-denatured ethanol for genetic analyses. Samples were sorted within a 3-4 days of collection.

Genetic Analyses

Each individual aquatic oligochaete was screened for myxozoan parasite DNA using a tiered extraction process with both Chelex® resin beads (Bio-Rad, Hercules, CA) and Qiagen DNeasy ® Blood and Tissue Kit (Qiagen, Hilden, Germany) being used. All samples were first extracted using Chelex®

beads . From the mid-section of each oligochaete, a pencil tip size sample was removed to be placed in the Chelex® solution.

Following Chelex® extraction, polymerase chain reaction (PCR) was completed using general myxozoan primers and targeting the 18S small ribosomal subunit (MyxF- ACC GTG GGA AAT CTA GAG CTA and MyxR – GTT CCA TGC TAT YAA CAT TCA A , Iwanowicz et al 2008) to screen oligochaetes for myxozoan parasites. Reaction volumes for PCR were 25ul with 12.5ul GoTaq® Green Master Mix 2x (Promega, Madison, WI), 1ul forward and reverse primer (5uM), 9.5ul reagent grade water, and 1ul template DNA. PCR programs followed the guidelines previously used in published papers for MyxF and MyxR (Iwanowicz et al. 2008, Walsh et al. 2012) including an annealing temperature of 52°C. After PCR, potential products were viewed by running a 2% agarose gel stained with GelGreen Nucleic Acid Stain (Phenix Research Products, Candler, NC). Any visible product was identified as a potential myxozoan positive sample. The samples identified as potential positives were then re-extracted with the DNeasy ®Blood and Tissue kit (Qiagen, Hilden, Germany) for long term DNA storage and additional analyses following the manufacturer's protocols.

After extraction with DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), PCR was then completed on the samples using the myxozoan general primers (MyxF and MyxR) and products were viewed on a 2% agarose gel as indicated above. Any positives from the re-extracted samples were also subjected to additional PCR to identify the aquatic oligochaete (invertebrate host). For molecular work on aquatic oligochaetes, primer set 16sar- CGC CTG TTT ATC AAA AAC AT and 16sbr- CCG GTC TGA ACT CAG ATC ACG T (Palumbi et al. 1991) was used and followed PCR conditions similar to previous uses (e.g., annealing 52°C; Pop et al. 2003). Positive samples were then prepped for sequencing. PCR cleanup was completed using Exonuclease 1 (Exo) (New England Biolabs, Ipswich, MA) and shrimp alkaline phosphatase (SAP) (New England Biolabs, Ipswich, MA). Following PCR cleanup, sequencing reaction was completed using the BigDye® Terminator v3.1 (Thermo Scientific, Waltham, MA) kit in both the forward and reverse direction. Following the sequencing reaction, the samples were ran through Centri-Sep™ (Princeton Separations, Freehold, NJ) filtration columns following the manufacturers protocol and dried. Dried samples were reconstituted with 25ul Hi-Di™ formamide (Thermo Scientific, Waltham, MA) and denatured prior to loading on the Applied Biosystems 3130xl Genetic Analyzer (Thermo Scientific, Waltham, MA) for sequencing. Contigs of the forward and reverse product were generated using Sequencher® (Gene Godes Corporation, Ann Arbor, MI) and a low complexity National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) was used to assess molecular identification.

Potential myxozoan positive samples on the second round of extractions were also tested with *Myxobolus inornatus* specific primers (513F- TCG ACG CCC TCC CTG ACT CG, 1312R- TGG ACG CTG CTG CGA ACA CC, 1438R- GTG CCA GCA GCC GCG GTA AT, Walsh et al. 2012). PCR programs followed guidelines of Walsh et al. (2012) including an annealing temperature of 63°C. A 2% agarose gel stained with GelGreen Nucleic Acid Stain (Phenix Research Products, Candler, NC) was used to view any products which were also subjected to sequencing if a product was visible.

Myxozoan parasite research 2015

During 2015, worms were collected and sampled in the same fashion as completed during 2014 sampling. Samples were collected between April –June 2015. Increased sampling intensity occurred at sites where worms were more prevalent in previous sampling years. In the West Branch of the Susquehanna River Basin, Pine Creek and Chillisquaque Creek were selected for continued sampling.

Contaminant Research 2014

Pilot work for contaminant analysis was completed during 2014 YOY sampling and included collecting juveniles from five sites in the West Branch of the Susquehanna River Basin. These sites were located within the telemetry study area except for one site (Loyalsock Creek). Samples were collected using backpack and towboat electrofishing in July 2014. In addition, site revisits were completed or later sampling was completed at two sites. One site on Pine Creek was sampled in July (Pine Ramsey A) and again in September (Pine Ramsey B) These sites overlapped with aquatic
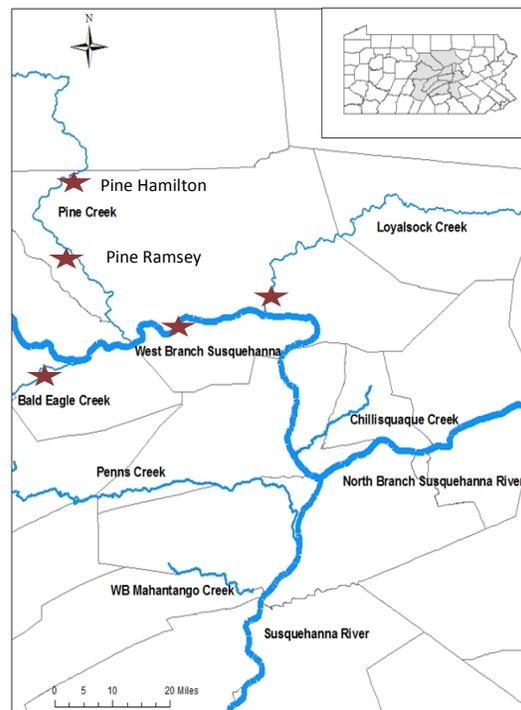

Figure 2: 2014 YOY contaminant sampling sites that were sampled within the West Branch of the Susquehanna River Basin (red stars). Two sites were located on Pine Creek, Hamilton Bottom and Ramsey. Sampling occurred in the central part of the state (see inset).

oligochaete sampling streams and including two sites on Pine Creek, one site on Bald Eagle Creek, one site on Loyalsock Creek, and one site on the West Branch of the Susquehanna River (Figure 2). A total of 10 individuals per site were targeted with fish collected from five different sites for contaminant analysis. After capture, fish were administered a lethal dose of tricaine methanesulfonate (MS22,

Tricaine-S, Western Chemical, Ferndale, WA), wrapped in foil, and placed on ice. Samples were frozen at -20°F until being sent in for analysis.

Samples were sent to the U.S. Geological Survey National Water Quality Laboratory located in Denver, Colorado and analyzed using the LC8079 tissue method. This method tests for 58 compounds including legacy compounds (e.g., PCBs, DDT, PBDEs), flame retardants, and a range of pesticides (e.g., cyhalothrin, cyfluthrin). A minimum one gram sample was needed for tissue analysis. The contaminant data that is quantified in the lab have both a minimum detection limit and a reporting limit. The reporting limit is the more conservative measure and is generally twice the minimum detection limit, although this also varies depending on the compound and calibration measures. Several compounds in the current schedule are also undergoing changes in some of these limits due to concerns with calibration and background contamination. With the advice from the lab, censored limits were increased for the following compounds polybrominated diphenyl ether (PBDE) 47, PBDE99, PBDE100, triclosan, and hexachlorbenzene to the minimum reporting limits (William Foreman, USGS, pers.comm.). These censored limits were based on calibration limits or levels of background measurement for the chemicals in the lab and varied for each specific compound. All remaining compounds are reported at the minimum detection limit or reporting limit as indicated.

Contaminant Research 2015

Contaminant work continued in 2015 and focused for this study within the telemetry study area. This complemented the larger effort of contaminant sampling which was also completed at several other sites within the Susquehanna River Basin. Similar to 2014, young of the year samples were collected from Bald Eagle Creek, Pine Creek, and the West Branch of the Susquehanna River during July. The same sampling methods were used as in 2014 for collection of juvenile fish, but additional steps were added to collection protocols including weighing fish and cleaning plastic sampling buckets with Liquinox® soap (Alconox, Inc., Orange, CA) and distilled water rinsing to prevent any background contamination. At each site the target was ten samples weighing one gram, which may have been more than ten individuals if individual fish needed to be pooled to meet the 1 gram weight requirement. Adult eggs were also collected for tissue analysis at the same sites where YOY were collected during pre-spawn collections (May-June 2015), with a target sample size of ten from each site. Fish were captured using boat electrofishing and a minimum one gram sample of female gonads was removed for contaminant analysis during fish health necropsies. Fish were administered a lethal dose of MS22 prior to conducting the necropsy. All tissue samples, both YOY and adult were immediately placed on ice and frozen at -20°F until shipping to the laboratory. The handling of fish in both 2014 and 2015 followed

protocols approved by The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC # 42544). In addition to tissue samples, sediment samples were collected during pre-spawn conditions (May-June 2015) and at the time of YOY collection (July 2015). These samples were collected following U.S. Geological Survey bed sediment sampling protocols (accessed online at: http://water.usgs.gov/nawqa/pnsp/pubs/ofr94-458/). All 2015 contaminant samples were sent to the U.S. Geological Survey Columbia Environmental Research Center (CERC) for analysis. The chemical analysis targeted a wide- variety of contaminants in both the sediments and tissue samples, including organochlorine pesticides, PCB and PBDE congeners (> 150 compounds total).

c.) **Results**

Myxozoan parasite research 2014

From the five sample sites, 506 worms were collected during 2014 sampling (Table 1). The majority of the worms were collected from Chillisquaque Creek (Table 1). Chillisquaque Creek had the largest proportion of suitable worm habitat, which included fine sediment and leaf litter

Table 1: Aquatic oligochaete samples collected at the five sites within the West Branch of the Susquehanna River Basin.

Site	Number of worms
Chillisquaque Creek	414
West Branch Susquehanna	5
Pine Creek	20
Loyalsock Creek	17
Bald Eagle Creek	50
Total	**506**

deposition areas. After preliminary extraction using Chelex® and secondary extraction with the DNeasy® Blood and Tissue kit, eleven samples produced a gel product using the myxozoan general primers (MyxF and MyxR) which cover variable regions one and two of the small ribosomal subunit. Two of the eleven samples did not produce clean sequencing products, and in the remaining nine samples there was a lack of high identity matches with NCBI BLAST searches for myxozoan sequences. (i.e., none of the identity matches were 99% or higher) (Table 2). *Myxobolus inornatus* , the parasite of interest, was not found as a top identity match in any of the samples, but was in the top four matches in four samples. However, the identity match was only in the 80-87% range. These four samples were retested with *M. inornatus* specific primers and one sample (C184) produced a PCR product. Sequencing of the product did not result in higher identity match to *M. inornatus.*

The samples that were potentially positive for myxozoan DNA (9 with quality DNA) were also sequenced to determine host identity (aquatic oligochaete) using 16sar and 16sbr. In the NCBI BLAST search, seven of the nine samples had the top match aligning with *Limnodrilus hoffmeisteri.* In the seven samples, four had identity matches of 99% with almost complete coverage of the sequence (query cover -99-100%). The other two samples had matches with other aquatic oligochaetes, but the match percentage and query cover were not as high (<93%). Although the matchers for *L. hoffmeisteri* were

relatively high, it is important to verify the molecular match with morphometric identification. These samples were sent to an aquatic invertebrate specialist for further confirmation, but results have not been received to date. The other two oligochaete species that were possible matches (*L. cervix* and *Sparganophilus* sp. L) had lower identity matches (92%) and morphometric analysis would need to be completed before these could be evaluated further.

Table 2: Sequencing results from the NCBI BLAST searches on the nine positive samples with clean sequence data. Both top aquatic oligochaete (worm) and myxozoan matches are given for each sample. For all the samples, the E values were 0.0 or close to 0.0 (e.g., 5.00e-118) which indicates the matches were not due to chance alone. * = samples that had *M. inornatus* within the top four matches.

Sample	Target	Primer	Species match	Accession #	Total score	Query Cover	% identity
C84*	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AF325977.1	791	93%	97%
	myxo	MF/MR	*Raabeia* sp. Type I	KJ152184.1	841	100%	91%
C122*	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AY885613.1	732	99%	93%
	myxo	MF/MR	*Triactinomyxon* spF	AF378351.1	1035	100%	97%
C184*	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AY885613.1	900	99%	99%
	myxo	MF/MR	*Raabeia* sp. type 1	KJ152184.1	699	98%	89%
C188	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AF325977.1	761	93%	96%
	myxo	MF/MR	*Myxobolus* sp. CMW-2004	AY591531.1	898	100%	97%
C295	worm	16sar/16sbr	*Limnodrilus cervix*	AF325984.1	660	93%	92%
	myxo	MF/MR	*Myxobolus* sp. CMW-2004	AY591531.1	946	99%	96%
C302*	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AY885613.1	898	100%	99%
	myxo	MF/MR	*Raabeia* sp. type 1	KJ152184.1	952	100%	95%
C335	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AY885613.1	918	98%	99%
	myxo	MF/MR	*Triactinomyxon sp. 'changzhou'*	JX477771.1	710	93%	89%
C448	worm	16sar/16sbr	*Limnodrilus hoffmeisteri*	AY885613.1	926	100%	99%
	myxo	MF/MR	*Myxobolus osburni*	AF378338.1	435	68%	85%
P498	worm	16sar/16sbr	*Sparganophilus* sp. L	JF267915.1	695	95%	92%
	myxo	MF/MR	*Myxobolus* sp. 2 HLE-2011	JF714995.1	874	98%	92%

Myxozoan parasite research 2015

During 2015, 482 aquatic oligochaete samples were collected from Chillisquaque Creek and Pine Creek. Again the majority of the samples were collected from Chillisquaque Creek (450). These samples are awaiting genetic analysis.

Contaminant research 2014- young of the year

A total of 34 YOY samples were sent for contaminant analysis from 2014 sampling. Due to the minimum sample weight requirements from the lab (minimum one gram), several of the samples had to be pooled for analysis (Table 3). Based on censoring and minimum detection limits, the West Branch of the Susquehanna River YOY samples had the largest number of contaminant detects (Figure 3). The remaining sites had relatively low number of detects (0-7), except for Pine Creek Hamilton Bottom, the upper Pine Creek site, which had a larger number of detects and also a larger range in the number of detects (1-13). Pine Creek Ramsey, which was sampled during two different time frames (A and B), had a relatively low number of detects both times, but a larger range in the number of detects during the second sampling event. Common compounds detected included triclosan (antimicrobial), polychlorinated biphenyl (PCB) 138 (persistent, insulators), PCB 180 (persistent, insulators), cyhalothrin (pyrethroid insecticide), dichlorodiphenyldichlorethylene (DDE)(degradate of persistent pesticide DDT), p, p', and trans-nonachlor (pesticide chlordane component) (Table 4). The finding of triclosan above the censored limit in 15 samples may be difficult to interpret given the laboratory concerns with background levels and wide range of potential sources of triclosan exposure (i.e., plastic containers). Therefore, any interpretation or speculation about the presence or sources of this compound should be done with caution.

Table 3: 2014 sample sites for YOY contaminant analysis included five sites within the West Branch of the Susquehanna River basin and one site revisit (Pine Creek A and B). Samples that were less than one gram had to be pooled for contaminant analysis as indicated below resulting in less than ten samples at most of the sites.

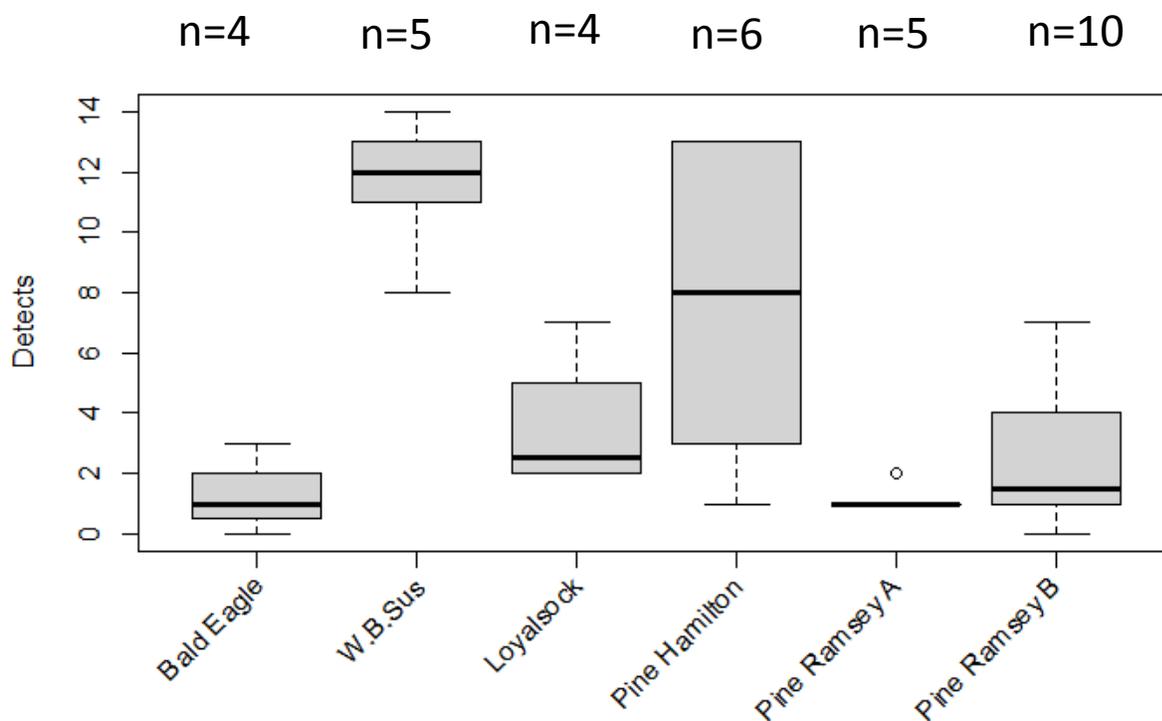| Site	Number of samples	Pooled
Bald Eagle Creek	4	Yes
West Branch Susquehanna River	5	Yes
Loyalsock Creek	4	No
Pine Creek Hamilton Bottom	6	Some pooled/some individual
Pine Creek Ramsey A	5	Yes
Pine Creek Ramsey B	10	No

Figure 3: The number of detected chemical compounds in 2014 YOY smallmouth bass for each site or time a site was surveyed. Boxplots represent the median (black line), first and third quartile (lower and upper limit of gray box), and range of the data by the whiskers. Outliers are denoted with a single point as shown in Pine Ramsey A. Pine Ramsey A and B are the same site sampled at two different time periods, July and September respectively.

Table 4: Out of the 58 compounds tested, six were commonly found above the reporting limit for 2014 YOY contaminant sampling. Number of detects indicates the number of times the compound was located above the reporting limit in the 34 samples. The predominant location indicates where this compound was most commonly found and in how many of those samples.

Compound	Number of detects	Predominant location
Triclosan	15	9/10 Pine Creek Ramsey B
PCB 138	11	All 5 West Branch Susquehanna
PCB108	6	All 5 West Branch Susquehanna
Cyhalothrin	6	3/6 Pine Creek Ramsey A
DDE, p,p'	6	2/4 Loyalsock, Pine Creek
Trans-nonachlor	5	All 5 West Branch

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2015 contaminant research

YOY, egg samples, and sediment samples were collected at the telemetry sites (Bald Eagle Creek, Pine Creek, and West Branch of the Susquehanna River) in 2015 and have been sent to the U.S. Geological Survey CERC laboratory for analysis. Target number of samples for YOY was collected from all three sites (10 samples), but adult egg samples only reached target numbers at Bald Eagle Creek (10). Pine Creek and the West Branch of the Susquehanna River sites had much lower number of females captured, 5 and 1 respectively. Although these samples have been sent into the lab for analysis, results have not been received to date.

d.) Conclusions

Myxozoan parasite research

The parasite *M. inornatus* was not detected in the aquatic oligochaete samples collected in 2014. Identity matches indicate that the myxozoans found may be related species, but there is no indication that any of the sequences were from *M. inornatus*. In a summary of previous work on myxozoans, molecular comparisons within species yielded anywhere from a 98 to almost 100% match in identity even when the same species was found in multiple hosts or separated geographically (Hallett et al. 2004). For example, *Kudo thrysites*, a myxozoan found to infect both atlantic salmon (*Salmo salar)* and tubesnout (*Aulorhynchus flavidust*), was less than 0.1% different when comparing the molecular sequences of the parasite from the two hosts (Hervio et al. 1997). If a species match was found for *M.inornatus,* it would be expected that the identity scores and query cover should both be close to 100% match. This did not occur and thus it is concluded that *M.inornatus* was not in the potential invertebrate host that were sampled.

The complex life cycle of myxozoans, having with both vertebrate and invertebrate hosts, adds complexity to the infection dynamics and elucidating timing of infection for the vertebrate host. YOY smallmouth bass collected at two to three months old in various parts of the Susquehanna River Basin have myxozoan infections, but little else is known regarding this parasite and the role it may play in other disease and mortality related concerns (Walsh et al. 2012, Smith et al. 2015). Myxozoan parasite infection can often be regarded as incidental findings of which the parasite does not result in host mortality or other disease mediated effects (Okamura et al. 2015). However, there are some well-known myxozoan parasites that have resulted in wide range mortality and disease events in a variety of fish species. For example, *Myxobolus cerebralis*, the causative agent of whirling disease in rainbow trout (*Onchrynchus mykiss)*, moves though nerves to infect cartilage which can result in fish mortality (El-Matbouli 1995). Similarly, *Ceratanova Shasta*, a myxozoan parasite affecting west coast salmon, has

resulted in ceratomyxosis (necrosis of gut lumen) and mortality with outbreaks in both the wild and the hatchery system (Hallett and Bartholomew 2011). Incidental parasite findings paired with other stressful environmental conditions (i.e., contaminants, bacteria, etc.) may also result in concerns for heightened disease, immunosuppression, and even mortality (Sitja-Babadilla et al. 2015). Thus, the research to date still leaves many questions regarding the role this parasite has, if any, in the onset of disease and mortality in juvenile smallmouth bass within the Susquehanna River basin.

Although *M. inornatus* was not isolated from the aquatic oligochaetes collected in 2014, DNA of other myxozoan parasites was found in a subset of worms collected. In general, there was not a species match (>98% identity with low evalue and high query cover) with these parasites and those found in the NCBI genomic database (GenBank: http://www.ncbi.nlm.nih.gov/genbank/). There were several greater than 95% identity, but without further analysis, it would be difficult to speculate further on the identity and relationship of these species. Some of the top myxozoan matches have been previously identified in invertebrate hosts include: *Triactinomyxon* sp. F, which was previously found in *Limnodrilus hoffmeisteri* in Ontario (Kent et al. 2001) and *Raabeia sp* Type 1, which was previously found in *Isochaetides michaelseni* in Hungary. One of the other top myxozoan matches has not been isolated in its invertebrate host, but has been identified in a vertebrate host. *Myxobolus sp.* CMW -2004 was previously found in the Willamette River in cyprinids, but other than the genomic database entry, these data have not been published elsewhere (Genomic Database entry: AY591531.1, Kent et al. 2005). To our knowledge, the other possible myxozoans found in the worms in the West Branch of the Susquehanna River have not yet been found in fish hosts in this region nor has their genomic data been entered in the GenBank for molecular comparison. However, it is possible that they are infecting vertebrate hosts other than the target species (smallmouth bass). For example, a closely related identity match for two of the aquatic oligochaete samples (96-97% identity match), *Myxobolus sp.* CMW-2004, was found to infect cyprinids. Without documentation of these myxozoans in fish hosts in the Susquehanna River Basin, further investigation is difficult to continue at this time. Having sequence information on these organisms may be important in the future if additional myxozoan infections in different fish species are identified. This may require additional sequencing to have larger sections of the 18S small ribosomal subunit.

The species of worm that was most commonly found as a possible host was *Limnodrilus hoffmeisteri,* which has been previously identified as a host for other myxozoan parasites (Hallett et al. 2005). Examples of myxozoan parasites that *L. hoffmeisteri* has served as an invertebrate host to include *Triactinomyxon sp.* Type 1 (Kent et al. 2001) and *Myxobolus parviformis* (Kallert et al. 2005). Although

M.inornatus was not identified in *L. hoffmeisteri*, this aquatic oligochaete may warrant additional investigation given its propensity to serve as an invertebrate host for other myxozoan species. It will also be important to continue to investigate a range of aquatic oligochaetes, including others that have previously been invertebrate hosts for myxozoan parasites. This includes other worms belonging to the family Naididae (this includes *L.hoffmeisteri)* which used to be known as Tubificidae (i.e., *Tubifex tubifex;* Ezterbauer et al. 2015).

One limiting factor in identifying the intermediate host may be related to sample sizes. In general, prevalence of myxozoan infection in aquatic oligochaetes is usually relatively low. For example, in sampling over 28,000 aquatic oligochaetes over a three year time period on Scotland, only 3.3% were found to be infected with myxozoan parasites (Özer et al. 2002). This indicates that a much larger sample size of aquatic oligochaetes may need to be sampled in order to get not only myxozoan positive samples, but a specific parasite of interest.

Contaminant Research

In general, there was a larger number of chemical compounds detected in the West Branch of the Susquehanna River than in the other tributary sites. This was not unexpected due to the fact that the river site may have many more anthropogenic influences than the tributary sites, including input from those tributaries (i.e., Pine Creek and Bald Eagle Creek are located upstream of the West Branch of the Susquehanna collection site). The upstream catchment of all of the sites are predominately forested (>70% forest; NLCD 2006, Fry et al. 2011), but the river site location was in close proximity to a town, Jersey Shore, PA, which is located next to the river and also receives input from all upstream tributaries on the West Branch of the Susquehanna River. Relatively low number of contaminant detects in the other sites could be due to the high percent forest found (>70%), relatively low agricultural land use in the catchment (<20%), and relatively low development (<10%) in the catchment (including all upstream influences). Local sources should also be considered especially where patterns or number of detects were not as expected. For example, the larger number of detects and variability at the Pine Creek Hamilton Bottom site warrants further investigation as the land use at both sites is relatively similar and predominately forested (>80% on both the local and catchment scale). In this instance, there may be other local landscape attributes that vary between the two sites, which at this time have not been identified.

The contaminant data from this project provides a baseline of information for future research. Several of the commonly found compounds in the YOY tissues are chemicals of concern in aquatic systems for a variety of reasons, including potential concerns with endocrine disruption and long term

persistence in the environment. For example, triclosan, an antimicrobial, has been shown to induce vitellogenin (yolk protein precursor) and alter sperm counts in male mosquito fish (Raut and Agnus 2010). In addition, triclosan mixed with another antimicrobial, triclocarban, was shown to alter behaviors important for nesting in male fathead minnows (i.e., defending territories, Schultz et al. 2011). Triclosan is also found in a wide range of products, from personal care products to plastics and textiles, which is resulting in it being commonly found in the environment (Schweizer 2001). Given that triclosan can have endocrine disrupting effects on fish, as shown in mosquito fish and fathead minnow (Raut and Agnus 2010, Schultz 2011), it may be important to quantify triclosan levels in fish tissue, while at the same time accounting for any background contamination that may occur due to its occurrence in so many materials.

Of the other compounds commonly detected, several are persistent compounds that take many years to break down in the aquatic environment and have the potential to bioaccumulate and biomagnify in aquatic organisms, including DDE, p,p', PCB congeners (PCB 138 and PCB 108), and chlordane mixture components including trans-nonachlor (Rasmussen et al. 1990, MacGregor 1973, Adu- Kumi et al. 2010). DDE, a metabolite of organochlorine pesticide DDT, was shown to increase in concentration in lanternfish off of California likely due to its persistent nature (MacGregor 1973). PCBs which were commonly used as insulators are also persistent in the environment. During the late 1970s and 1980s concentration of PCBs in fish tissue in Lake Ontario was biomagnified in the food chain (Rassmussen et al. 1990). Trans-nonachlor and other chlordane pesticide constituents have also accumulated in aquatic organisms. For example, catfish and tilapia surveyed from lakes in Ghana were found to have a wide range of persistent contaminants including DDT and its metabolites as well as chlordane components, including trans-nonachlor (Adu-Kumi et al. 2010). Due to their persistent nature, it was not unexpected to find some of these compounds in YOY smallmouth bass.

In addition to potential for accumulation in organisms, many of these persistent compounds also have a wide range of potential detrimental effects on living organisms. Several PCB congeners, including PCB 138 found in young of the year smallmouth bass in this study, have been shown to cause damage to DNA in fish cell lines (Marabini et al. 2011). PCBs have also been found to have endocrine disruptive properties in fish which may differ depending on the orientation of chlorine molecules (co-planar vs. non co-planar) (Caló et al. 2010). Both agonistic and antagonist effects on vitellogenin production in fish were noticed when exposed to different PCB congeners (Caló et al. 2010). Of the other persistent compounds, DDE, p,p' and trans-nonachlor have both been shown to have estrogenic properties including altering sex ratios in turtles which resulted in increased numbers of female when the eggs

were exposed to chemicals (Willingham and Crews 1999). Endocrine disruption in populations raises the concern for future reproductive effects that may result, including declines in reproductive potential or altered behavior of individuals (i.e., decreases in sperm or changes in spawning behaviors; Raut and Agnus 2011, Schultz et al. 2011).

Other than the persistent chemicals and the antimicrobial triclosan, the other commonly found compound, cyhalothrin, is a pyrethroid pesticide which usually breaks down in presence of light and is not very stable in water, especially at pH levels above 8 (He. et al. 2008). Although it may not be as persistent as some of the other chemicals, it has been shown to have detrimental effects on development, growth, and defense enzymes in juvenile carp (Richeterova et al. 2014). Thus, there is a wide range of potential detrimental effects from the contaminants found in YOY smallmouth bass, although it is difficult to determine what consequences these chemicals may have as often times it can be difficult to predict how chemicals act in mixtures versus individual exposures (Jordan et al. 2011). In addition to contaminant effects or concerns alone, other stressors, including parasites, may mediate or alter fish immune response (Marcogliese and Pietrock 2011). Thus it may be difficult to predict the outcome of exposure to both pathogens and chemicals, and this may warrant future investigative studies in a laboratory setting to separate out how fish respond to both individual and combined stressors. Future laboratory trials or exposures could also focus on integrating environmentally relevant conditions including contaminant concentrations found in the fish tissue.

Without having data from 2015, it is difficult to draw further connections between YOY contaminants and the contaminants found in adult gonads and the surrounding environment. This makes it difficult to consider adult movement dynamics and potential contaminants present in adult gonads. The 2015 data, which tested for a greater number of contaminants, may also allow for a more thorough investigation of the contaminants present in young of the year tissue.

e.) Additional Research Indicated

After reviewing 2014 data, there still are a lot of uncertainties and questions that remain. However, data from the samples collected in 2015, once back from the lab, should help inform the types and concentrations of chemicals found in smallmouth bass and their habitats. There is also a lot of additional ongoing work related to both the myxozoan parasite work and contaminant work which is detailed below:

1.) *Myxozoan Parasite Research* - For the myxozoan parasite work, the invertebrate host has not yet been identified and still little is known about *M.inornatus* prior to infecting the fish host.

a.) **2015 aquatic oligochaete samples** – These samples have not yet been processed, but is proposed to occur over the next few months. However, the number of aquatic oligochaete samples may not be enough to detect *M.inornatus* in its host. Thus, an evaluation of the number of samples needed and an investigation into alternate ways to collect samples may be warranted.

b.) **qPCR assay to detect spores in water samples** - In conjunction with aquatic oligochaete sampling, a qPCR assay is also being developed by M. Schall to detect and quantify actinospores (infective agent released from invertebrate host) of *M.inornatus* in the aquatic environment. If the parasite can be detected from the water samples, it will be possible to gain more information on the timing of infection. Water samples were collected and filtered from three sites in the Susquehanna River Basin in 2015. Assay development is currently underway.

c.) **Myxozoan prevalence in YOY** – Since 2013, YOY have been collected and a subset have been removed for histological analysis to screen for myxozoan parasites from several sites within the Susquehanna River Basin as well as out of the Susquehanna River Basin. Prevalence of myxozoan parasites will be compared at sites where samples were collected as well as over multiple years of data collected at the same site when available. This research is part of a larger collaborative effort to pair ongoing myxozoan research with various fish health initiatives.

2.) *Contaminant Research*-Similarly, the contaminant research for this project complements ongoing research at other sites in the Susquehanna River Basin. Some of the additional ongoing work is listed below:

a.) **2015 contaminant data for this project** – Currently, 2015 contaminant results have not been received from the laboratory. These data, once received, will provide more insight on contaminants not only in YOY, but also in the adult eggs and sediment. Since this research also occurred in the telemetry study area, where we have an understanding of fish movement patterns, it may be possible to get an idea of different potential sources of exposure to contaminants given movement patterns. Additionally, the laboratory used for the 2015 analysis tests for a much larger number of compounds which will allow for more contaminants to be investigated.

b.) **Other 2015 contaminant sampling**- In addition to the contaminant work for this research, a larger collaborative contaminant sampling also has been, and is occurring,

throughout the Susquehanna River Basin. This includes, but is not limited to, collection of adult and juvenile smallmouth bass samples, sediment samples, and water samples for contaminant analysis.

3.) *Addendum to this report* – Because 2015 data has either not been analyzed (2015 aquatic oligochaete samples) or not yet been received from the laboratory (2015 contaminant samples), an addendum to this report will be provided once the data are analyzed.

f.) Acknowledgements

g.) Citations

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1. **Appendix A**
    a. **Staff**
        i. **Number of individuals**
            1. 1- PhD candidate - Megan Kepler Schall
            2. 1- Advisor to PhD candidate – Dr. Tyler Wagner – U.S. Geological Survey, Pennsylvania Cooperative Fish and Wildlife Research Unit, Adjunct Professor at Pennsylvania State University
            3. 3-PSU technicians – Lydia Neal, Tyler Thompson, Stephen Sbrolla
            4. State and Federal Agency and University Assistance – several individuals from various organizations provided assistance during the grant including individuals from Pennsylvania Fish and Boat Commission, Pennsylvania Department of Environmental Protection, U.S. Geological Survey, Susquehanna River Basin Commission and, Pennsylvania State University, and Susquehanna University.

        ii. **Number of full-time employees (as part of the grant)** – This funding did not provide wages or funding for salary of employees.

        iii. **Number of full-time employees (as part of match)** – No match provided

    b. **Students Supported**
        i. **Number of Undergraduate Students**- Several undergraduate students from Pennsylvania State University (Tyler Thompson, Stephen Sbrolla) and Susquehanna University (Laurel Seemiller, Nathan Newton) assisted during the project, but were not supported by this grant.

        ii. **Number of Graduate Students**-1 PhD candidate - Megan Kepler Schall

        iii. **Number of Ph.D. Students** -1 – Megan Kepler Schall

        iv. **Degrees Awarded** (please indicate level)- Megan Kepler Schall is currently a PhD candidate in the Intercollege Graduate Degree Program in Ecology at Pennsylvania State University with an anticipated graduation date of December 2017.

    c. **Outreach/Extension**
        i. **Number of meetings, workshops, or conferences, and number of attendees**
            1. *AFS Fish Health Section Meeting* – June 2015, Title: Investigation of contaminants and disease characteristics of young of the year smallmouth bass in the Susquehanna River basin, PA. Approx. number of attendees – 30-40.

        ii. **Number of public or professional presentations, and number of attendees**
            1. *Susquehanna University: Guest Lecture* – February 2015. Title: Investigation of risk factors affecting population status of Susquehanna River smallmouth bass. Approx. number attending: 20

2. *Mansfield University: Guest Lecture* –March 2015. Title: Investigation of risk factors affecting Susquehanna River smallmouth bass. Approx. number attending: 20-30.
3. *Penn State Ecology Graduate Student Organization* –*Science Café.* March 2015. Title: Wild animals get sick too! Approx. number attending: 30-40
4. *California University of Pennsylvania: Guest Lecture* – March 2016, Title: Investigation of risk factors affecting population status of Susquehanna River smallmouth bass. Approx. number attending: 20

2. **Appendix B**

Impact Statement(s)

1. Relevance: This work supplements other ongoing research efforts on smallmouth bass in the Susquehanna River basin by providing insight on contaminants and parasites as risk factors for young of the year smallmouth bass disease and mortality. Collaboration with multiple agencies and partners continues to strengthen the research efforts and also aligns with many of the research needs set forth in the Susquehanna River CADDIS report (http://www.dep.pa.gov/Business/Water/PointNonPointMgmt/WaterQuality/Pages/SusquehannaRiverStudy.aspx#.VpZC1vkrJaS), including studying both contaminants and parasites.

2. **Response**: This research provides initial information on contaminants present in young of the year smallmouth and also further exemplifies the difficulty in elucidating parasite-host relationships. In the future, with more contaminant data and research on parasites, the data summarized in this report will provide information for managers and researchers to 1.) identify contaminants of interest that are accumulating in fish tissues, 2.) identify potential sources of contaminants (i.e., sediment, maternal egg sources), 3.) allocate research efforts to priority areas based on contaminant findings at different sites, and 4.) begin to understand the complex interactions in parasite–host-contaminant relationships.

3. **Results:** This research will be pertinent to use for the design of future research projects as well as to help inform management decisions. For example, gaining a baseline understanding of what chemicals are being stored in fish tissues will be important for identifying compounds of concern and also possibly setting threshold levels for future regulations and consumption advisories. This research, when combined with other ongoing collaborative efforts to measure a variety of water quality, fish health, and fish population dynamics, will contribute towards a comprehensive evaluation of the smallmouth bass populations in the Susquehanna River basin.

4. **Project Partners**: This project was possible through the collaboration with a wide range of partners including the following Pennsylvania Fish and Boat Commission, Pennsylvania Department of Environmental Protection, U.S. Geological Survey, Pennsylvania State University, Susquehanna University, and Susquehanna River Basin Commission.