Flounder Monitoring Report:
2015 Results

Massachusetts Water Resources Authority

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Submitted to

Massachusetts Water Resources Authority
Environmental Quality Department
100 First Avenue
Charlestown Navy Yard
Boston, MA 02129
(617) 242-6000

prepared by

Michael Moore[1]
Anne E. McElroy[2]
Paul Geoghegan[3]
Matthew R. Siskey[2]
Ann E. Pembroke[3]

[1]Woods Hole Oceanographic Institution
Woods Hole, MA 02543

[2]Stony Brook University
Stony Brook, NY 11794

[3]Normandeau Associates, Inc.
25 Nashua Road
Bedford, NH 30110

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EXECUTIVE SUMMARY

The detection of high prevalence of contaminant-associated liver disease such as liver tumors and centrotubular hydropic vacuolation (CHV) in winter flounder from Boston Harbor in the late 1980s was one of the findings that contributed to the concern about the ecological health of the Harbor. For example, in 1988, over 75% of flounder collected in Boston Harbor showed evidence of disease in liver tissue known to be associated with contaminant exposure, and 12% of the fish contained neoplasias (liver tumors), which also have a contaminant related etiology (Moore et al. 1996).

The siting of MWRA's Massachusetts Bay outfall caused concerns that flounder in Massachusetts Bay exposed to the relocated effluent discharge might over time show substantially increased prevalence of these contaminant-associated lesions. Therefore, a long-term monitoring program for winter flounder was established. The goals of this program are to provide data that can be used to assess potential impacts to winter flounder in the vicinity of the outfall and to track the expected long-term improvements in flounder health in Boston Harbor. Flounder are collected from near the outfall and from sites in Boston Harbor, off Nantasket Beach, and in Cape Cod Bay. Flounder from each site are sampled annually for length, weight, age, biological condition, and the presence of external or internal disease. Concentrations of inorganic and organic contaminants in body tissues are determined every third year, including in 2015. Contaminant concentrations in flounder, lobster, and mussel tissues will be evaluated in a forthcoming report.

Liver histology. The 2015 data represent the fifteenth consecutive year of flounder monitoring since the start-up of the Massachusetts Bay outfall in September 2000. Results of the histological analyses in 2015 support previous observations made from this long-term dataset.

- The mildest form of liver lesions (disease) associated with exposure to contaminants is CHV). During the 1980s, more than 75% of the flounder collected at Deer Island Flats showed evidence of the lesion. Over time CHV prevalence decreased from over 75% in 1988 to approximately 32% or less in recent years. This is a remarkable change.

- The high liver tumor prevalence characteristic of fish from Deer Island Flats in the mid- to late-1980s has disappeared. Neoplasia has not been observed in a fish from Boston Harbor since 2004, and has never been observed in fish collected at the outfall site.

- The above trends notwithstanding, in 2015, flounder from Deer Island had a relatively high prevalence of CHV, which while lower than was observed when the discharge was in Boston Harbor, was higher than that observed since the early 2000s.

- The prevalence of CHV in flounder from the vicinity of MWRA's Massachusetts Bay outfall has not shown increases over levels observed during baseline monitoring. During most years since offshore discharge was initiated, prevalence has been less than that observed during the baseline monitoring before 2001. CHV prevalence did increase consistently between 2005 and 2010, and has generally declined since then, with some year-to-year variability (Figure 3-12).

Evaluation of female-skewed sex ratios. When the results of the flounder monitoring were discussed in 2014 with regulators, the Outfall Monitoring Science Advisory Panel, and the public, questions were raised about the high percentages of female flounder seen in MWRA's data, which have approached 100% in some years at some stations (e.g. Figure 3-5). An evaluation of these and similar data was conducted for this report. First is an evaluation of the variability in the percent females in the MWRA data, looking for spatial and temporal trends. Second is an evaluation of the MWRA data in context of similar data collected by regional fisheries management agencies and other studies. Third is a discussion of these results and summary of other relevant research. Highlights of the findings from these evaluations are described below.

- The spatial and temporal evaluation of sex ratios in flounder from Boston Harbor and the Bays confirms that the large adult flounder sampled by MWRA have substantially female-skewed sex ratios, with 83% female flounder when averaged over all sites and all years.
- Regression analyses designed to identify changes in temporal trends in the sex ratios document that for all MWRA monitoring stations, sex ratios were less female-skewed early in the monitoring program than in recent years.
    - For flounder caught near the current MWRA outfall this difference is small, with 86% female in early years, and 89% females more recently.
- There was no consistent pattern between stations in the temporal trends identified or in the timing of the change in trend.
    - For flounder caught on Deer Island flats, there was no apparent trend in the data (for example, consistent increase or decrease) from 1991 to 1995, compared to data from 1996 to 2015, but the percent females increased from 63% in the early years to 91% in later years.
    - For fish caught near the outfall, there was a detectable increasing trend in the percent females caught between the start of monitoring in 1991 and 1999, and a detectable **decreasing** trend in the percent females between 2000 and 2015.
    - For flounder caught in Cape Cod Bay, farthest from the historical discharges in Boston Harbor and the current MWRA outfall, there was a detectable increasing trend in the percent females between 1991 and 1996, with no trend in the sex ratios since 1997 and, on average, the highest percent females in MWRA's monitoring data.
    - There was no identifiable association between the trends identified or the timing of the change in trends that could be associated with changes to MWRA's treatment or outfall start-up.

Evaluations were made comparing MWRA's data to those from other large surveys evaluating winter flounder in coastal waters from Maine to New Jersey. Most of these studies looked at flounder with a greater range in size than those sampled by MWRA, whose monitoring targeted larger, older fish in which exposure to contaminants in the environment has had time to result in liver disease, and many evaluated fish caught at different times of the year. To maximize data comparability, only data on spring caught fish of 30 cm total length were compared among surveys.

- All of the coastal populations evaluated show a trend of increasing percent female fish with size, and all show strongly female-skewed sex ratios for large fish.
- For all coastal studies (including MWRA's) in all years, fish greater than 30 cm total length, the minimum size included in MWRA's study, average 83% female.
- Data from studies conducted for flounder caught from north of Cape Cod to the New Hampshire border by the Massachusetts Division of Marine Fisheries show a temporal trend in percent females very similar to that seen in MWRA's data.
- Temporal trends in sex ratios observed in flounder from Massachusetts are not present in all the coastal data sets evaluated here. Winter flounder from New Jersey show a decreasing trend in the percent females, and there is no temporal trend in percent females in studies of fish from Connecticut, Rhode Island, and Maine/New Hampshire.
- In contrast to all coastal surveys, flounder from Georges Bank do not show substantial female-biased sex ratios, and there is no marked increase in the percent females with age in that population.

There are a number of factors that can influence sex ratios in fish; among them are environmental factors like temperature as well as the potential exposure to chemicals that can act to mimic or block the action of endogenous hormones. This study was not able to suggest what factors might be associated with the female-skewed sex ratios found in large (30 cm+ total length) flounder from coastal populations in recent years, especially north of New Jersey, or why offshore fish in the Georges Bank population do not demonstrate similar sex ratios. However, the similarity in sex ratios observed in state surveys of inshore waters from northern Maine to Connecticut does not really support hypotheses implicating either environmental factors or contaminants, as temperature and likely chemical contamination vary substantially over this area. Given the very strong correlation between sex and size in coastal populations of adult winter flounder, factors associated with temporal changes in the size of flounder found at these sampling sites are likely responsible for the patterns observed. These patterns were not observed in the Georges Bank winter flounder possibly because this is a completely different genetic stock with different relationships between growth rate, size and sex ratios.

Even without comparing the MWRA data to data collected by the other state-wide monitoring programs, a link between the increasing trend in female biased sex ratios and sewage derived contaminants being released from the Boston outfalls (either old or new) would have been unlikely. Female-skewed sex ratios that increased between early in the monitoring and in recent years were observed at all four of the MWRA monitoring sites, including reference sites off Nantasket Beach and Eastern Cape Cod Bay. Contaminant impact from Boston Harbor or Massachusetts Bay sources is implausible unless there was almost perfect mixing of flounder throughout the area. Such mixing is highly unlikely given the persistent, stable differences in liver pathology prevalence recorded between the different MWRA stations, as seen in Section 3 of this report. Such differences would not be present if the fish mixed through the study area.

Collectively, all three lines of evaluation reported in this study point to the absence of a link between sewage releases into Boston Harbor and Massachusetts Bay and female biased sex ratios.

ACKNOWLEDGMENTS

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Connecticut Department of Environmental Management

Maine Department of Marine Resources

Massachusetts Division of Marine Fisheries

National Oceanic and Atmospheric Administration – National Marine Fisheries Service

New Jersey Department of Fish and Wildlife

University of Rhode Island, Graduate School of Oceanography

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1. INTRODUCTION

The Massachusetts Water Resources Authority (MWRA) has implemented a long-term Harbor and Outfall Monitoring (HOM) Program for Massachusetts and Cape Cod Bays. The objectives of the HOM Program are to test whether the environmental impacts of the MWRA discharge are consistent with SEIS projections and do not exceed Contingency Plan thresholds (MWRA 2001). A detailed description of the monitoring and its rationale is provided in the Effluent Outfall Monitoring Plan developed for the baseline period and the post-discharge monitoring plan (MWRA 1997, 2004, 2010).

The detection of high prevalence of contaminant-associated liver disease (a condition known as centrotubular hydropic vacuolation) in winter flounder from Boston Harbor in the late 1980s was one of the findings that contributed to the concern about the ecological health of the Harbor. For example, in 1988, over 75% of flounder collected in Boston Harbor showed evidence of disease in liver tissue associated with contaminant exposure, and 12% of the fish contained liver tumors, also associated with exposure to contaminants (Moore et al. 1996).

Following the design of the MWRA Deer Island Treatment Plant and the siting of the Massachusetts Bay outfall, concerns were raised that flounder in Massachusetts Bay exposed to the relocated effluent discharge might over time show substantially increased prevalence of these contaminant-associated lesions. Therefore, a long-term monitoring program for winter flounder (MWRA 1991) was established. The goals of this program are to provide data that can be used to assess potential impacts to winter flounder in the vicinity of the outfall and to track the expected long-term improvements in flounder in Boston Harbor. Resident flounder are collected from near the outfall and from sites in Boston Harbor, Massachusetts Bay, and Cape Cod Bay (hereafter: Boston Harbor and the Bays). Measured parameters for flounder include length, weight, age, biological condition, the presence of external or internal disease, and concentrations of inorganic and organic contaminants in body tissues. Data have been collected since 1992. The full program was conducted annually until 2003. Since then tissue contaminant monitoring is now done every third year (for example, in 2015). Flounder morphology and histopathology remain on an annual schedule.

The first sections of this report present morphology and histopathology results for the 2015 flounder survey. The evaluations are focused on assessing changes to flounder condition that may have resulted from improved wastewater treatment and the relocation of the outfall discharge. The 2015 data represent the fifteenth consecutive year of flounder monitoring since the start-up of the Massachusetts Bay outfall in September 2000. A summary of the survey and laboratory methods used for flounder monitoring is provided in Section 2. The results of monitoring data from the survey conducted during 2015, along with comparisons to historical flounder data, are presented in Section 3.

When the results of the flounder monitoring were discussed in 2014 with regulators, the Outfall Monitoring Science Advisory Panel, and the public, questions were raised about the high percentages of female flounder in MWRA's data, which has approached 100% in some years and some stations (e.g. Figure 3-5). Section 4 of this report contains an in-depth evaluation of the flounder gender data in MWRA's collections in comparison to other, similar regional datasets.

Finally, conclusions drawn from the 2015 results and historical trends are summarized in Section 5. By comparing values with established thresholds and evaluating trends over time, these flounder data are used to ensure that discharge of effluent into the Bay does not result in measured adverse impacts to fish.

2. METHODS

Winter flounder (*Pseudopleuronectes americanus*) were collected from four locations in Boston Harbor and the Bays (Figure 2-1) to obtain specimens for age, weight, and length determination, gross examination of health, and histology of livers. The methods and protocols used during the 2015 flounder survey were similar to and consistent with previously used methods. Detailed descriptions of the methods are contained in the Quality Assurance Project Plan (QAPP) for Fish and Shellfish Monitoring 2014–2016 (Geoghegan et al. 2014).

2.1 Stations and Sampling

The 2015 flounder survey was conducted between April 27 and May 7, 2015. Four sites were sampled to collect winter flounder for histological analyses:

- Deer Island Flats (DIF)
- Off Nantasket Beach (NB)
- Outfall Site (OS)
- East Cape Cod Bay (ECCB)

Figure 2-1 shows the monitoring locations. Table 2-1 provides the planned and actual sampling sites and locations for the 2015 flounder sampling.

Otter-trawl tows were conducted from the F/V *Harvest Moon* operated by Captain Mark Carroll. The scientific crew consisted of principal investigator Dr. Michael Moore from WHOI, and biologist Eric Rydbeck from Normandeau.

Mobilization for the survey was conducted on April 27th, when 8 fish were collected from Deer Island and 50 from each of Nantasket Beach and then on April 28th 50 were collected from the Outfall Site and 3 more from Deer Island. On April 29th 50 fish were collected from Eastern Cape Cod Bay. On May 7th, 39 fish were collected from Deer Island Flats. Fish were weighed and measured individually in the field. Scales and otoliths were removed from each fish for aging and livers were removed, sliced, examined and three slices fixed. Internal examination and tissue sampling, including sampling for histology and chemistry, was completed onshore by Envirosystems, Inc., for 15 fish from each station. Results of the contaminant chemistry analyses will be evaluated in a subsequent report.

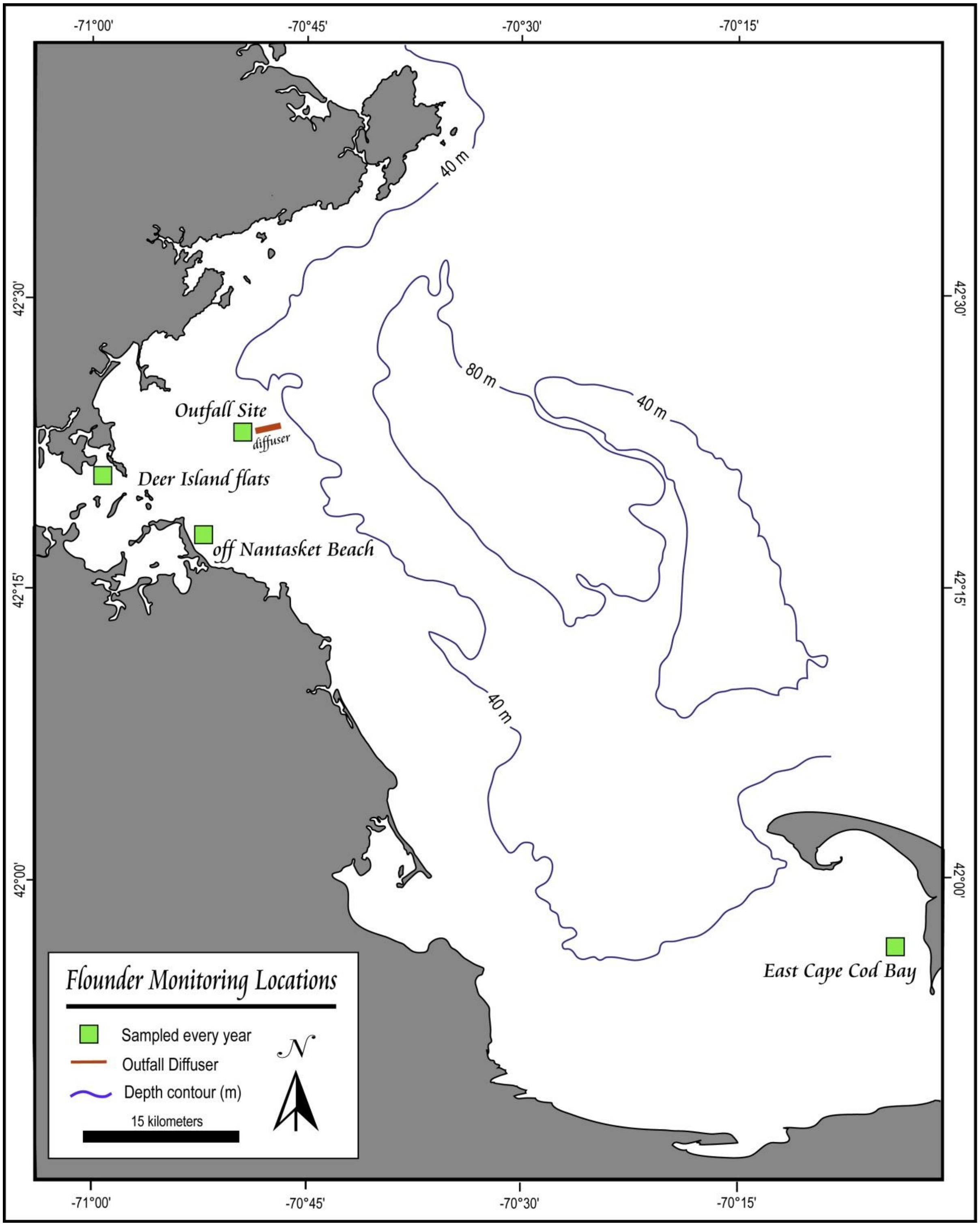

Figure 2-1. Flounder monitoring locations for 2015.

Table 2-1. Flounder Sampling Locations in 2015.

Site (Station ID)/Date/Time			Actual Location		Planned Location	
			Latitude	**Longitude**	**Latitude**	**Longitude**
Deer Island Flats (DIF)	**27-Apr-15**	**7:28**	42.3467	-70.9647	42.34	-70.9733
		9:05	42.3485	-70.9663	42.34	-70.9733
		10:03	42.3475	-70.9653	42.34	-70.9733
	28-Apr-15	**12:46**	42.3485	-70.9669	42.34	-70.9733
	7-May-15	**8:15**	42.3496	-70.9675	42.34	-70.9733
		8:54	42.3521	-70.9723	42.34	-70.9733
		10:00	42.3518	-70.9728	42.34	-70.9733
East Cape Cod Bay (ECCB)	**29-Apr-15**	**9:00**	41.941	-70.1262	41.9367	-70.11
		10:52	41.9283	-70.126	41.9367	-70.11
Off Nantasket Beach (NB)	**27-Apr-15**	**11:25**	42.2911	-70.8692	42.2933	-70.87
		12:37	42.2822	-70.8613	42.2933	-70.87
		13:48	42.2896	-70.8675	42.2933	-70.87
Outfall Site (OS)	**28-Apr-15**	**9:48**	42.3829	-70.825	42.385	-70.8217

2.2 Histological Analysis

Livers of 50 flounder from each site were prepared for histological analysis by Experimental Pathology Laboratories in Herndon, VA. Transverse sections of flounder livers fixed as part of tissue sample processing were removed from the buffered formalin after at least 24 hours, rinsed in running tap water, dehydrated through a series of ethanols, cleared in xylene, and embedded in paraffin. Paraffin-embedded material was sectioned on a rotary microtome at a thickness of 5 µm. Each block contained three liver slices, resulting in one slide with three slices per slide per fish, for a total of 200 slides (50 fish x 4 sites). The sections were stained in hematoxylin and eosin. Each slide was examined by Dr. Moore under bright-field illumination at 25 x, 100 x, and 200 x magnification to quantify the presence and extent of

- Three types of vacuolation (centrotubular, tubular, and focal)
- Macrophage aggregation
- Biliary duct proliferation and trematode parasitism
- Neoplasia

The severity of each lesion was rated on a scale of 0 to 4, where: 0 = absent, 1 = minor, 2 = moderate, 3 = severe, and 4 = extreme.

2.3 Data Reduction and General Data Treatment

All fish data (1991 to 2015) were extracted directly from the HOM database and imported into SAS (version 9.2), where data reduction, graphical presentations and statistical analyses were performed. Data reduction was conducted as described in the Quality Assurance Project Plan (QAPP) for Fish and Shellfish Monitoring 2014–2016 (Geoghegan et al. 2014). For each liver lesion and each fish, a histopathological index was calculated as a mean of scores from three slices on one slide.

Histopathological indices and prevalence of lesions were compared among groups of flounder by differences in station and age. Flounder monitoring parameters were presented graphically and compared among stations and over time.

2.4 Deviations from the QAPP

The NOAA Age and Growth Laboratory were unable to age 29 of the fish using otoliths due to ‘otoliths being broken, or processing issues.' A request for NOAA to use scales for those fish in lieu was not practical, given that the lab now lacks anyone with that skill set. The scales were therefore aged by Normandeau Associates.

3. RESULTS AND DISCUSSION

3.1 Fish Collected

Winter flounder, each a minimum 30 cm in length, were collected between April 27 and May 7, 2015, at four stations in the study area (Figure 2-1). The catch per unit effort (CPUE), defined as the number of fish at least 30 cm long obtained per minute of bottom trawling time, is reported per station in Figure 3-1. Effort was constant up to and including 2007 with the FV Odessa (70' sweep rope). For 2008, the FV Harvest Moon (74' sweep rope) was used for DIF, NB, and OS, with a net that was 1.04 x wider and for ECCB the FV Explorer 2 (84' sweep rope) was used with a net that was 1.2 x wider. Since 2009, the FV Harvest Moon has been used for all stations. Thus, data presented in Figure 3-1 have been normalized to the Odessa sweep length by using the ratio of sweep lengths as a multiplier (i.e., CPUE's for the FV Explorer net were multiplied by 70/84, and CPUE's for the Harvest Moon net by 70/74, to get CPUE units in Odessa equivalents). CPUE in 2015 was lower compared to 2014 at all stations except for OS (Figure 3-1). CPUE was highest at ECCB and OS, and lowest at DIF and NB.

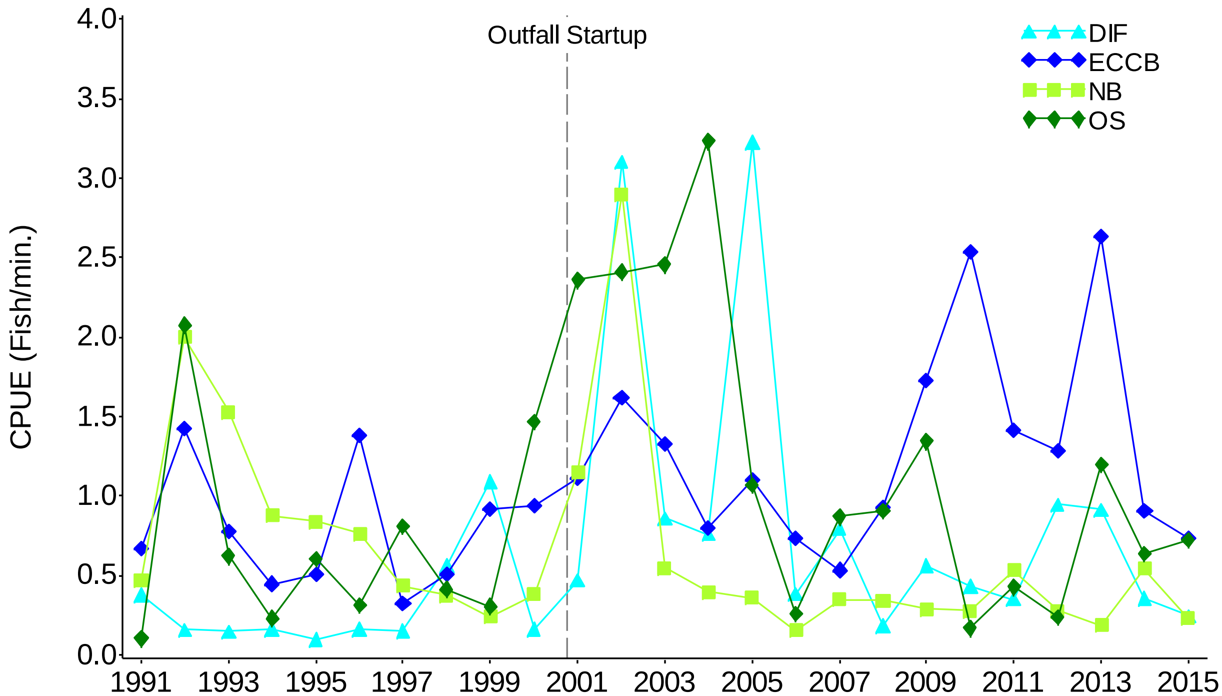

Figure 3-1. Catch Per Unit Effort (CPUE) for winter flounder trawled 1991–2015. Data for 2008 to 2015 have been normalized (see Section 3.1).

3.2 Physical Characteristics

Mean values for physical characteristics of the winter flounder collected in 2015 are reported in Table 3-1. These values reflect the project requirement to collect sexually mature specimens (>30cm total length). Mean age ranged from 4.1 to 5.0 years across the stations. Mean standard length ranged from 274 to 292 mm and mean total length from 335 to 357 mm; weight ranged from a mean of 427 to 518 g.

Mean age in 2015 compared to 2014 (Figure 3-2) was less for all stations. 2014 was the first year when all age data were determined from otoliths, which is considered a more robust method for age determination than the previously used method of scale analysis. Comparisons between the two methods indicate that for older fish the otolith method may provide an older age than the scale method. Compared to 2014, standard length (Figure 3-3) in 2015 showed a slight decrease for DIF and OS and a slight increase for NB and ECCB. Note the use of scales again for some fish in 2015. Weights (Figure 3-4) decreased at DIF and increased at ECCB, OS and NB. Percent females (Figure 3-5) increased at OS, and decreased at DIF, ECCB and NB.

Table 3-1. Summary of Physical Characteristics of Winter Flounder Collected in 2015.

Parameter	DIF			ECCB			NB			OS		
	Mean	STDDEV	N	Mean	STDDEV	N	Mean	STDDEV	N	Mean	STDDEV	N
Age (years)	4.16	1.45	49	5.02	1.48	50	4.12	1.44	50	5.02	1.95	50
Standard Length (mm)	292.3	29.7	50	290.54	23.32	50	279.06	20.57	50	273.76	20.94	50
Total Length (mm)	357	36.61	50	352.28	28.77	50	340.46	24.62	50	334.52	25.35	50
Weight (g)	517.5	160.12	50	501.6	140.61	50	463.1	120.56	50	426.7	116.49	50

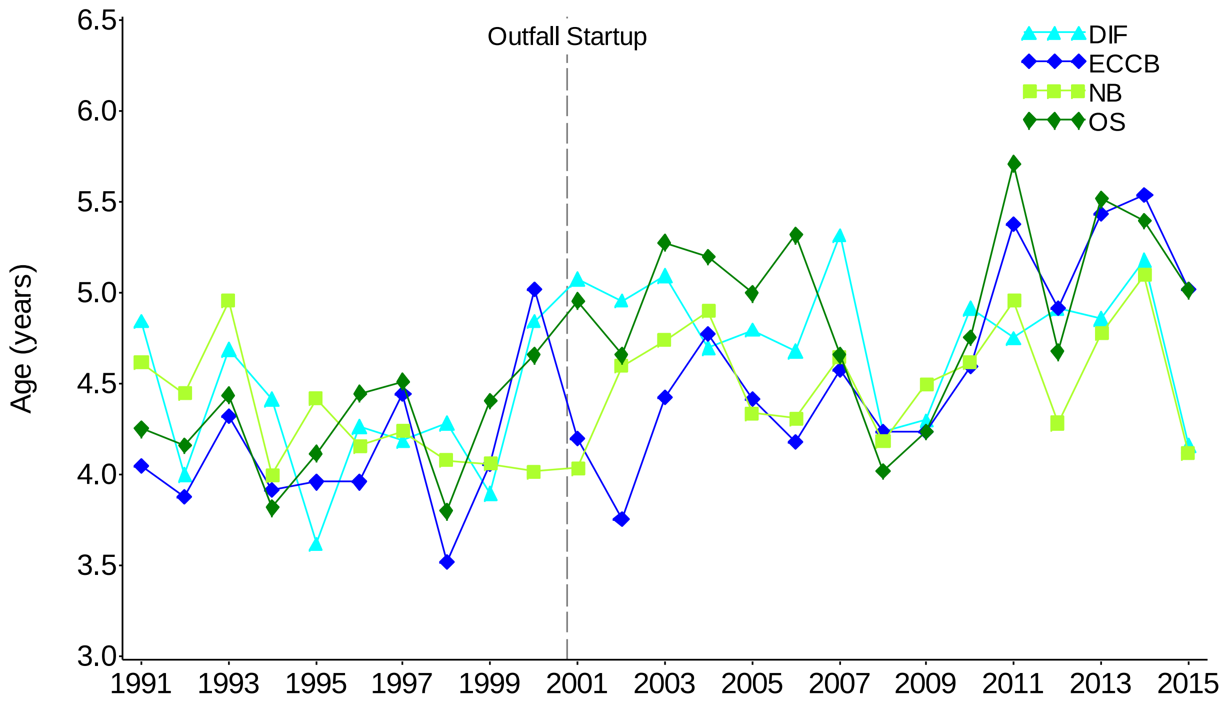

Figure 3-2. Average flounder age (years) compared by station and year.

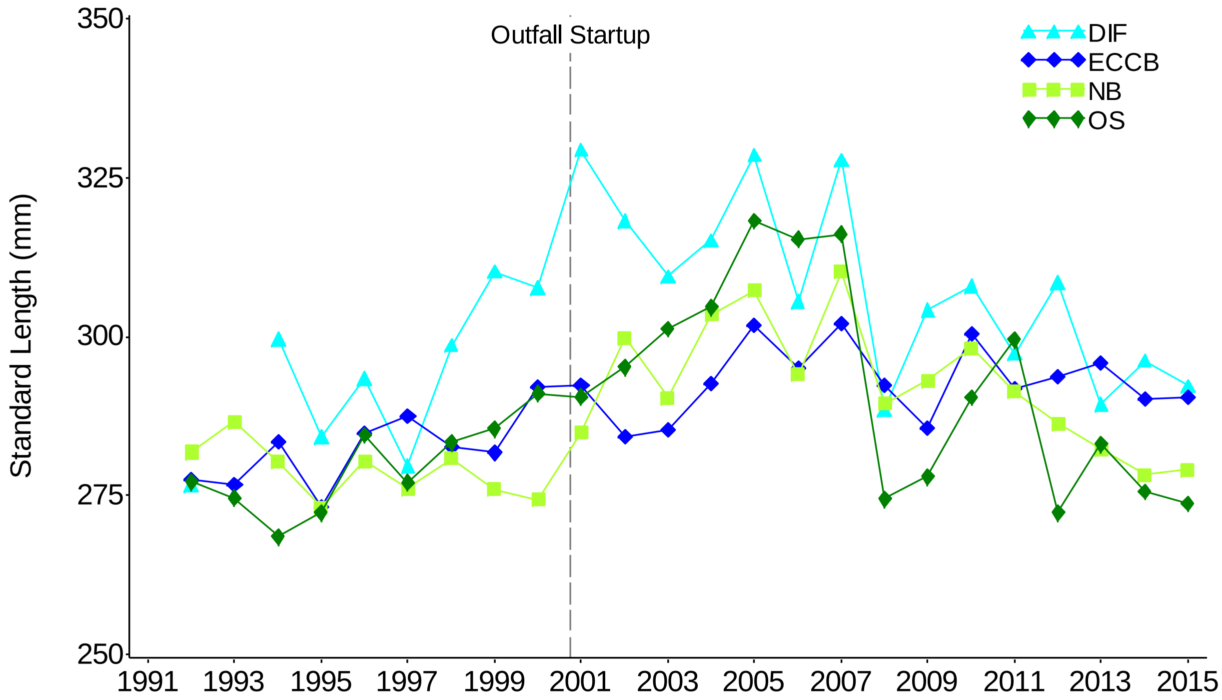

Figure 3-3. Average flounder standard length (mm) compared by station and year.

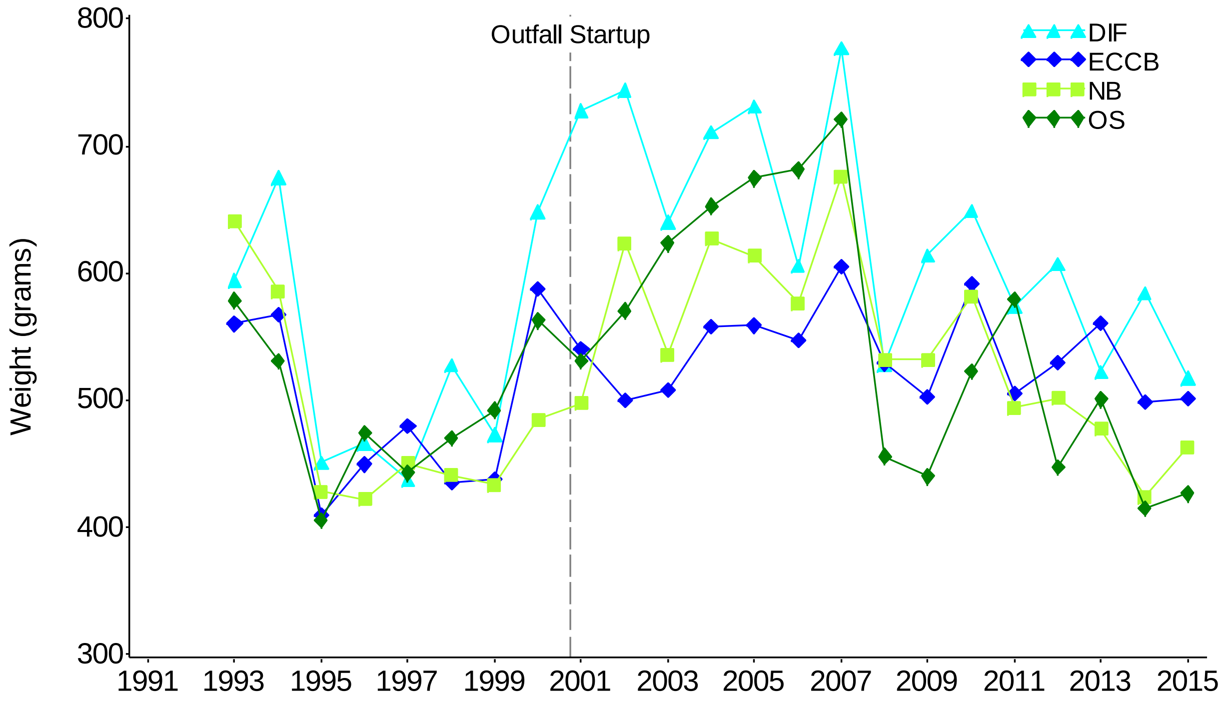

Figure 3-4. Average flounder weight (grams) compared by station and year.

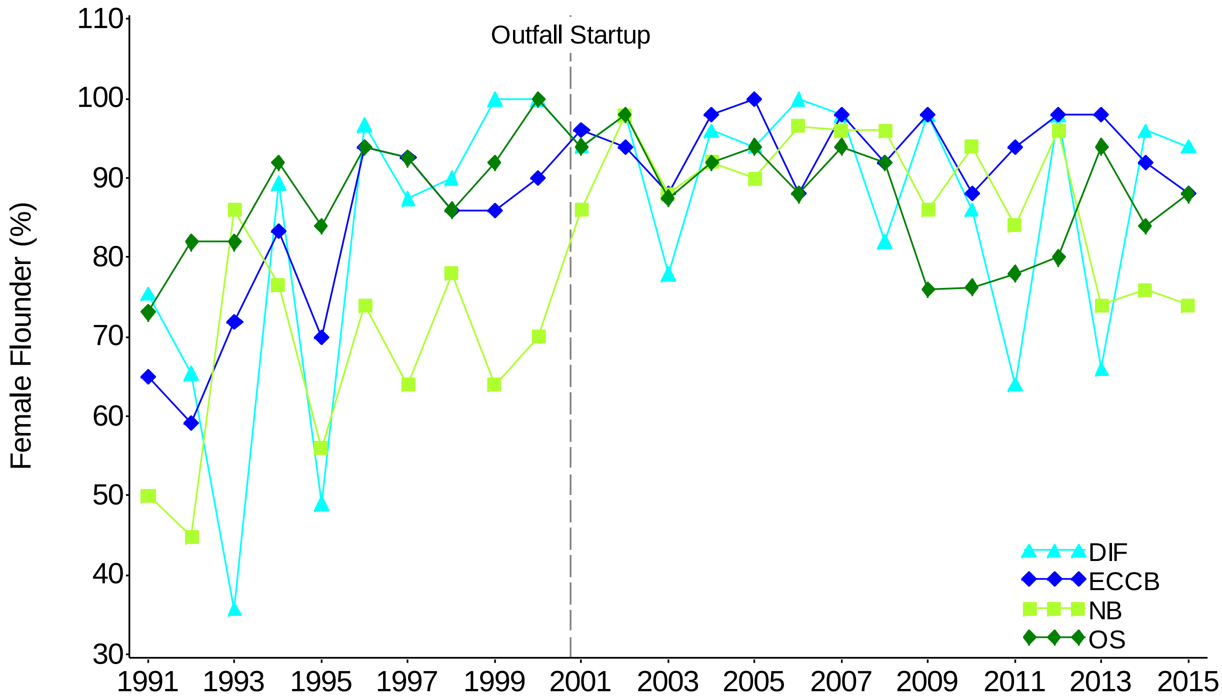

Figure 3-5. Proportion of female flounder compared by station and year.

3.3 External Condition

The external conditions of winter flounder collected in 2015 are presented as prevalence (% of individuals) per station in Table 3-2. Bent fin ray ranged from 2 to 20%, being highest at DIF.

Table 3-2. Prevalence (%) of External Conditions Assessed for Winter Flounder Collected in 2015.

External Conditions	Station (Sample size)			
	DIF (50)	ECCB (50)	NB (50)	OS (50)
Bent Fin Ray	20	14	2	6
Blind Side Ulcers	4	0	2	2
Fin Erosion (Fin Rot)	20	20	22	4
Lymphocystis	32	30	38	66

Ulcer prevalence has been recorded since 2003. It is unclear if ulcers were absent prior to 2003, given lack of a specific record, but if they were present, it was at a very low level. Elevated levels of ulcers were observed from 2003-06, then decreased from 2007-10, and were once again elevated in 2011 (Figure 3-6). In 2014, 2% were ulcerated at DIF and OS. Blind side ulcers were absent on all fish at ECCB in 2015, and were found on one fish each from NB and OS (2%) and on 2 fish (4%) at DIF. Fin erosion ranged from 4 to 22%, being highest at NB. Lymphocystis ranged from 30% at ECCB to 66% at OS.

Fin ray surface mucous and epithelia are impacted by increased levels of ammonia and other pollutants, making fin erosion a useful parameter for detecting deteriorating water quality conditions (Bosakowski and Wagner 1994). The prevalence of fin erosion for each year was calculated for each station and plotted in Figure 3-7. In 2015 fin erosion decreased at DIF and OS remained the same at NB and increased at ECCB.

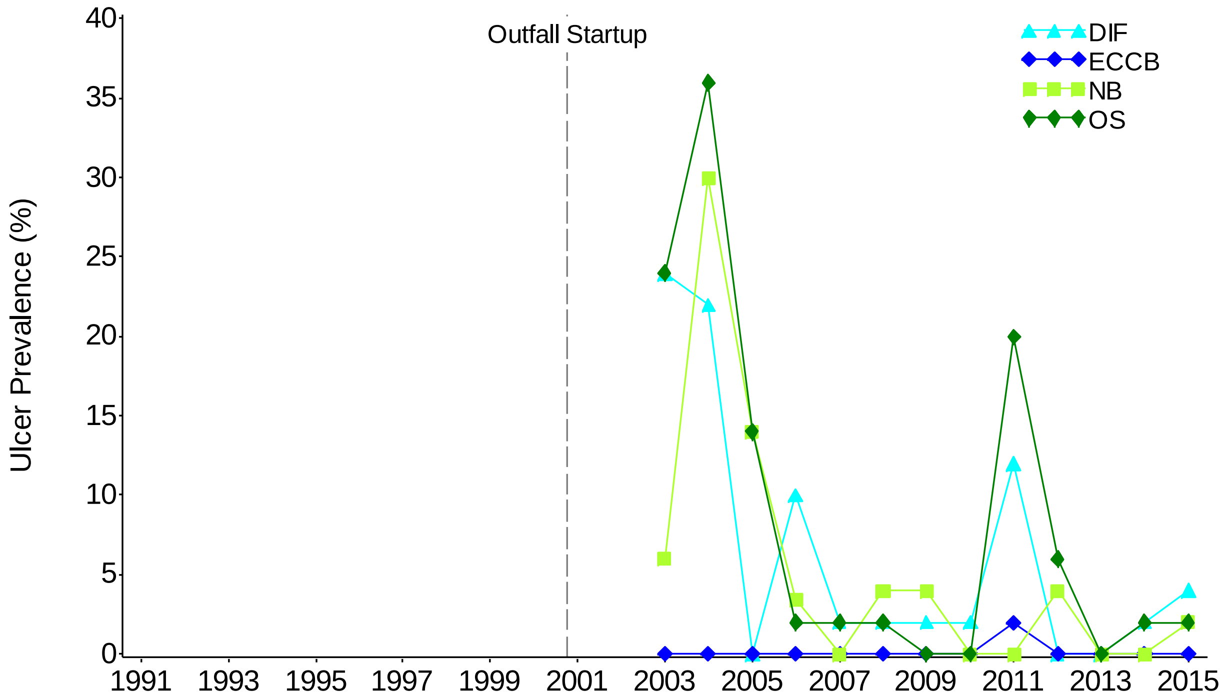

Figure 3-6. Temporal comparison of blind side ulcer prevalence (%) in winter flounder by station.

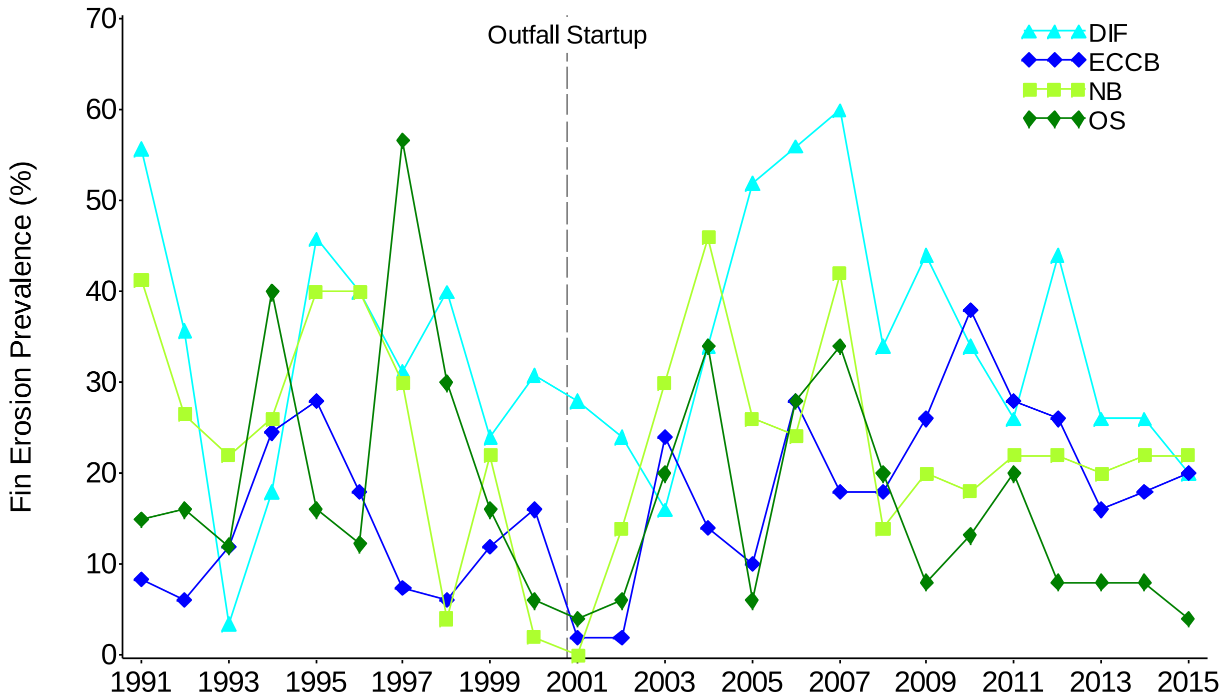

Figure 3-7. Temporal comparison of fin erosion prevalence (%) in winter flounder by station.

3.4 Liver Lesion Prevalence

The prevalence (% of individuals) of liver lesions in winter flounder from each of the four stations sampled in 2015 is presented in Table 3-3. Balloons ranged from 4 to 28%, bile duct protozoa were absent at all stations, biliary proliferation ranged from 4 to 6%, centrotubular vacuolation ranged from 4 to 32% being lowest at ECCB and highest at DIF, focal hydropic vacuolation was absent at all sites, and

Table 3-3. Prevalence (%) of Liver Lesions in Winter Flounder Collected in 2015.

Liver Conditions	Station (Sample size)			
	DIF (50)	ECCB (50)	NB (50)	OS (50)
Balloons	4	16	28	12
Bile Duct Protozoan	0	0	0	0
Liver Flukes	0	2	0	0
Biliary Proliferation	6	4	6	6
Macrophage Aggregation	60	42	64	62
Centrotubular Hydropic Vacuolation	32	4	10	10
Tubular Hydropic Vacuolation	24	0	0	4
Focal Hydropic Vacuolation	0	0	0	0
Neoplasia	0	0	0	0

liver flukes ranged from 0 to 2%. Macrophage aggregation ranged from 42 to 64%, neoplasia was absent at all sites, and tubular hydropic vacuolation ranged from 0 to 24%.

Neoplasms (Figure 3-8) remained absent at all sites, a situation that has persisted since 2005. Thus it continues to be true that the most significant histopathology associated with flounder from Boston Harbor before the MWRA project began remains totally absent.

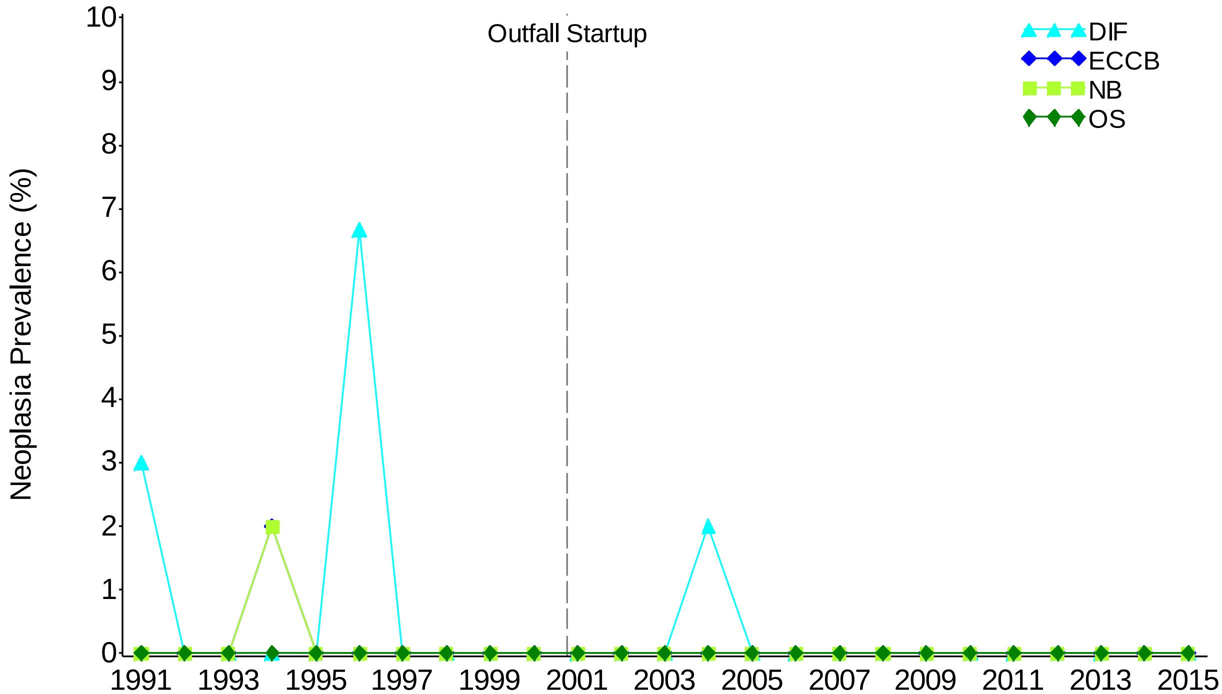

Figure 3-8. Temporal comparison of neoplasia prevalence (%) in winter flounder by station.

Along with neoplasms, hydropic vacuolation, because of its relationship to environmental contaminants, has been one of the principal lesions monitored in winter flounder throughout the program. Figure 3-9 shows centrotubular hydropic vacuolation at Deer Island Flats in 2015 was at the highest level in nearly a decade. It remains to be seen if this trend continues. An ongoing gentle increase at OS since 2005 decreased in 2012, increased again in 2013, decreased in 2014 and remained level in 2015; 2015 levels at OS are now below all of the pre-discharge years. A continued gentle low-level increase at NB through 2012 decreased in 2013, then increased again in 2014, and then decreased in 2015. A background level continued at ECCB, although it crept up a bit in 2013, fell to zero again in 2014, and then increased in 2015.

The severity of CHV (Figure 3-10) has also generally declined since 1991 at DIF, although there was a marked increase in 2015 at that station. Severity at DIF has typically been higher than the intermediate levels found at NB and OS. During 2015, severity was comparable at NB, and OS, with continued background low levels at ECCB.

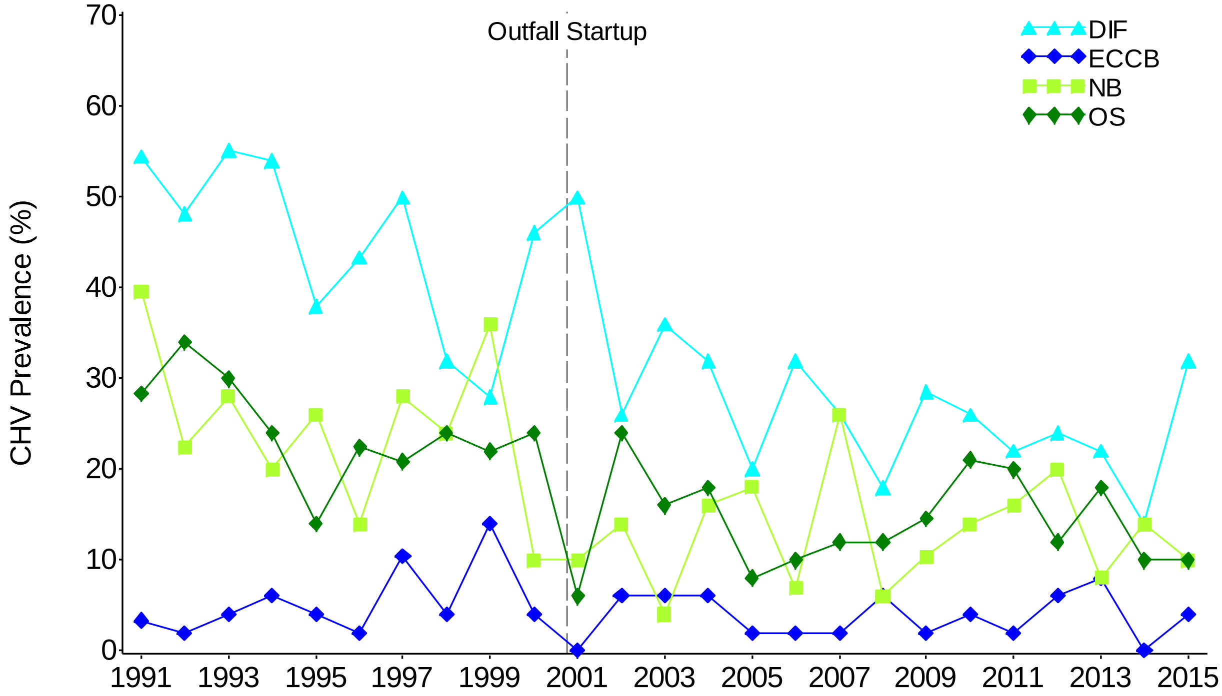

Figure 3-9. Temporal comparison of prevalence (%) of centrotubular hydropic vacuolation in winter flounder by station.

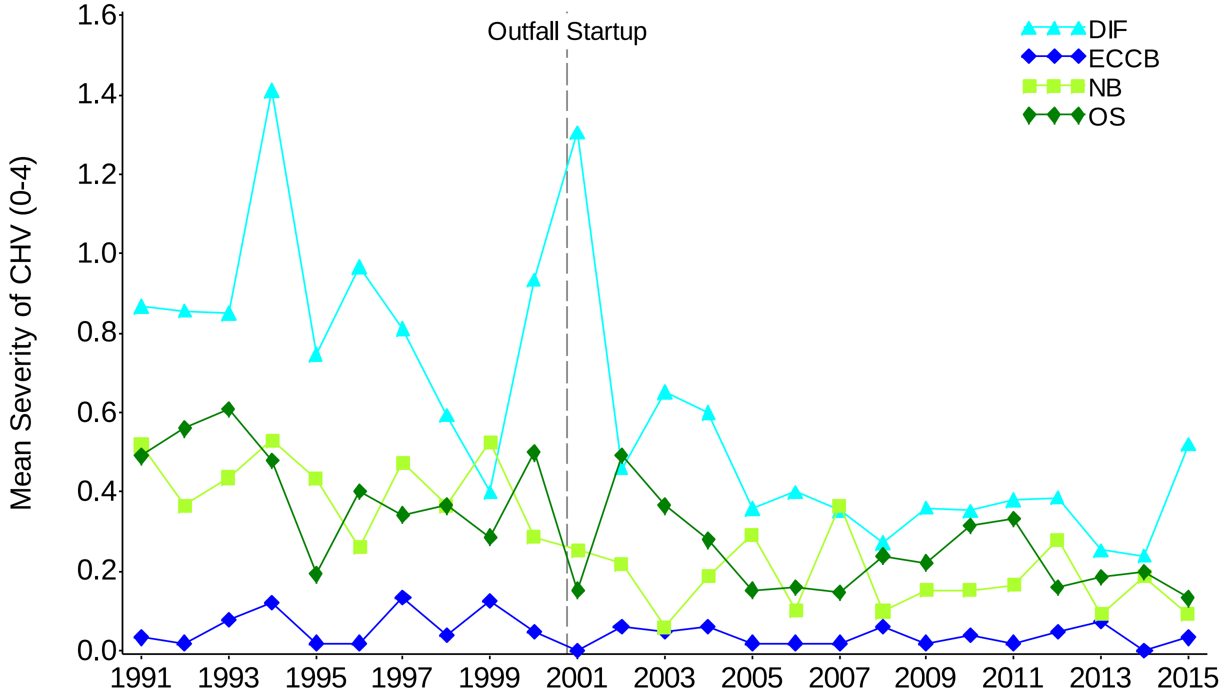

Figure 3-10 Centrotubular hydropic vacuolation severity (rank) in winter flounder compared between sites and years.

Relationships between age and lesion prevalence were also analyzed. The proportion of fish that had CHV (using data collected since 1997) was calculated for each age class at all stations (Figure 3-11). A modest increase in CHV, as might be expected with increased age, was found at ECCB. DIF shows a greater increase with age during baseline monitoring, compared to after the outfall was diverted offshore, suggesting a reducing cumulative impact of remaining toxicants thought to induce this lesion. A slight increase with age is perhaps also seen at OS during baseline monitoring. OS after diversion appears to be close to stable with age.

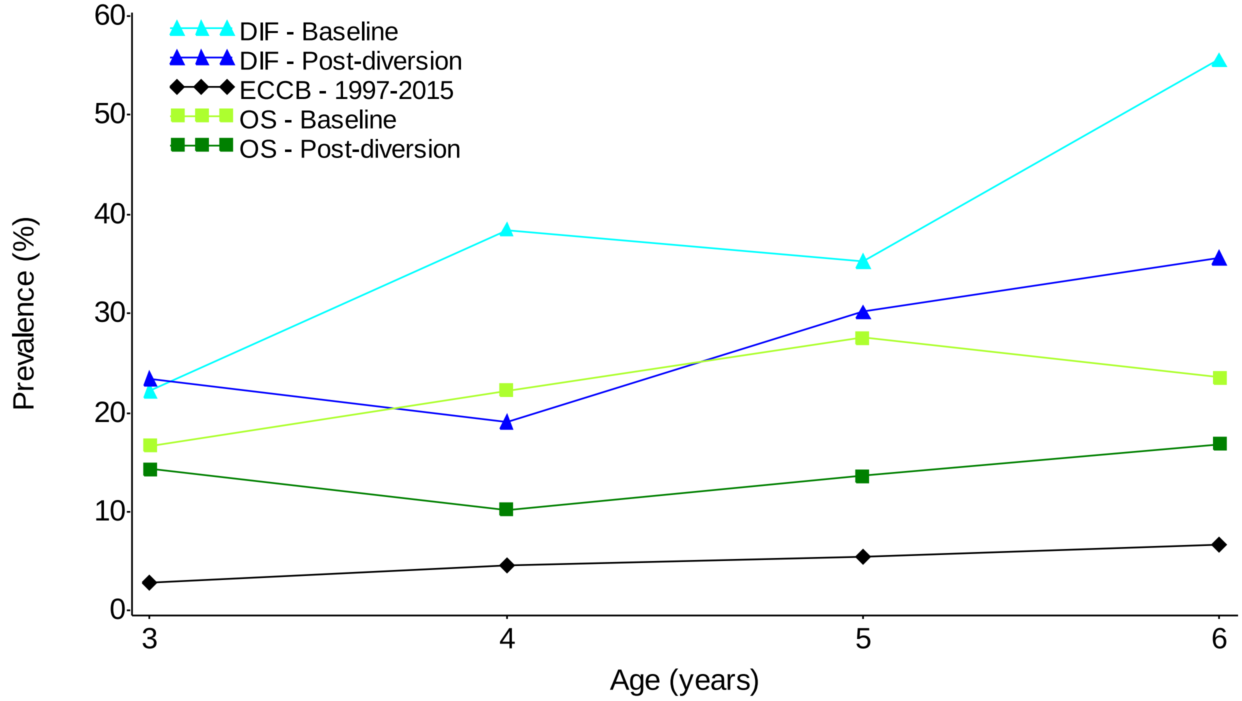

Figure 3-11. Proportion (%) of winter flounder showing hydropic vacuolation for each age.

To further assess the impact of changes in age on hydropic vacuolation prevalence, the percentage of fish at each station in each year that showed some degree of hydropic vacuolation was divided by the average age of fish for that year at that station. This generated an age-corrected index for the presence of hydropic vacuolation (Figure 3-12). The overall downward trend for DIF showed a mild increase in 2012 that then decreased again in 2013 and 2014, but then increased in 2015. In 2013 OS increased following a 2011/2012 decrease: this went down again in 2014 and then up in 2015. NB has shown recent increases from 2008-2012 that decreased in 2013 then increased again in 2014 and then decreased in 2015, and ECCB remains at historic background levels.

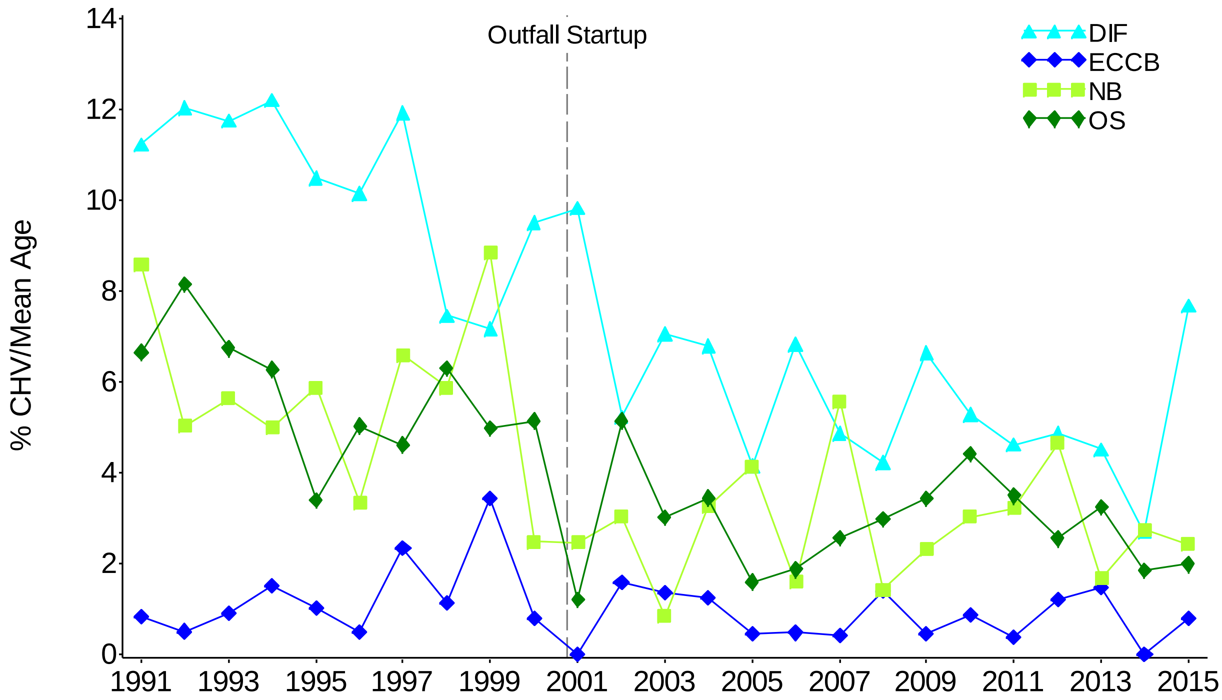

Figure 3-12. Hydropic vacuolation index (CHV%/age) for each station by year.

3.5 Threshold Comparison

The MWRA Monitoring Program has established threshold levels against which to measure key indicators of wastewater impacts (MWRA 2001). Because of the concerns that effluent discharge might increase the prevalence of lesions in Massachusetts Bay populations of winter flounder towards levels seen in Boston Harbor in the 1980s, liver disease prevalence was selected as a key indicator, with a Caution Level threshold set at 44.94% for the prevalence of centrotubular hydropic vacuolation (CHV) in winter flounder collected at the Outfall Site. CHV prevalence at the Outfall Site during 2015 was 10%, well below the threshold level.

4. TRENDS IN GENDER RATIOS

The preponderance of female flounder in MWRA's monitoring data in recent years has raised questions regarding the cause. This Section contains an evaluation of the female-skewed gender ratios found in winter flounder from this project. First, an evaluation is made of the trends in percent female winter flounder in the MWRA data. Second is an evaluation of the MWRA data in comparison to similar data collected by regional fisheries management agencies and other studies. Lastly is a discussion of these results and summary of other relevant research.

Limitations in Existing Data – In evaluating the trends in gender ratios, it is important to recognize that neither the MWRA flounder monitoring program nor the other programs from which data were evaluated were specifically designed to determine population-wide gender composition in flounder. The MWRA monitoring studies target fish longer than 300 mm total length to focus on flounder old enough so that exposure to contaminants in the environment has had enough time to result in contaminant-associated liver lesions (See Section 4.2.1). Thus MWRA's monitoring program has no data on younger fish. Stock assessment programs sample smaller (and younger) flounder than in the MWRA program, but as described in Section 4.2.1 their collections are also not intended to provide robust estimates of the sex ratios of flounder on a population wide basis. Therefore, the data are not unbiased estimates of sex rations in flounder at a population level. Despite these limitations, the project team is confident the data from different studies are reasonably comparable and provide gross representations of winter flounder sex ratios from several different monitoring programs.

4.1 Patterns in MWRA Data

Segmented regression was used to provide an objective interpretation of the trends in the MWRA winter flounder data. Segmented regression is a statistical technique that identifies the point in a data set where the relationship between the dependent and independent variables changes abruptly. In this analysis, the time series of four parameters (percentage females, standard length, weight, catch per unit effort) related to the MWRA winter flounder data set were examined. While a visual examination of these time series may identify one or more breakpoints in the data depending on who does the examination, segmented regression provides an objective description of where a time series changes significantly and provides exploratory analysis to identify trends in the data. The software SegReg (http://www.waterlog.info/segreg.htm) was used to perform these analyses with the intent of identifying significant trends in the data and years when these trends may have changed. Additional details regarding segmented regression are found on the SegReg website.

4.1.1 Percentage of Females

The most important parameter examined was the percentage of females captured at each of the winter flounder sampling stations. The sex of winter flounder was determined in the field by visual examination of gonads (Geoghegan *et al*. 2014). Statistical analyses of percentage data can be biased because it is a ratio, and the denominator (number of fish examined) and numerator (number of females) can both vary. Because the years are equally weighted in the regression, a given year where very few fish were examined can have a larger influence on the results than warranted. However, in this analysis the number of fish examined at each station and year ranged from 26 to 167. Therefore, the regression results were not unduly influenced by a few years with a very small number of fish examined.

The percentage of female winter flounder at Deer Island Flats changed significantly between 1995 and 1996 (Table 4-1, Figure 4-1). There were no trends in either of the segments of the time series, indicating an increase in percentage of females that started in 1996. The period 1995 and earlier had a lower average percentage of female winter flounder (63%) compared to 1996 and later (91%). However, in 1995 only 16 fish were collected in April and 34 were collected in May (Mitchell et al., 1996). The collection of flounder later in the year may have influenced the sex ratio in 1995.

Percentage of female winter flounder in East Cape Cod Bay also increased with time (Table 4-1, Figure 4-2). From 1991 through 1996, there was a significant positive trend in percentage female winter flounder with and average percentage of 74%. For the period 1997 and later, there was no significant trend but the average percentage of female winter flounder was higher than the previous period (93%).

At the Nantasket Beach there was a positive trend in the percentage of female winter flounder from 1991 through 2002 with an average of 71% female (Table 4-1, Figure 4-3). There was no trend from 2002 through 2015 and the average percentage of female winter flounder was 88%. The result was an increase in the percentage of females though 2002 and then no trend after that.

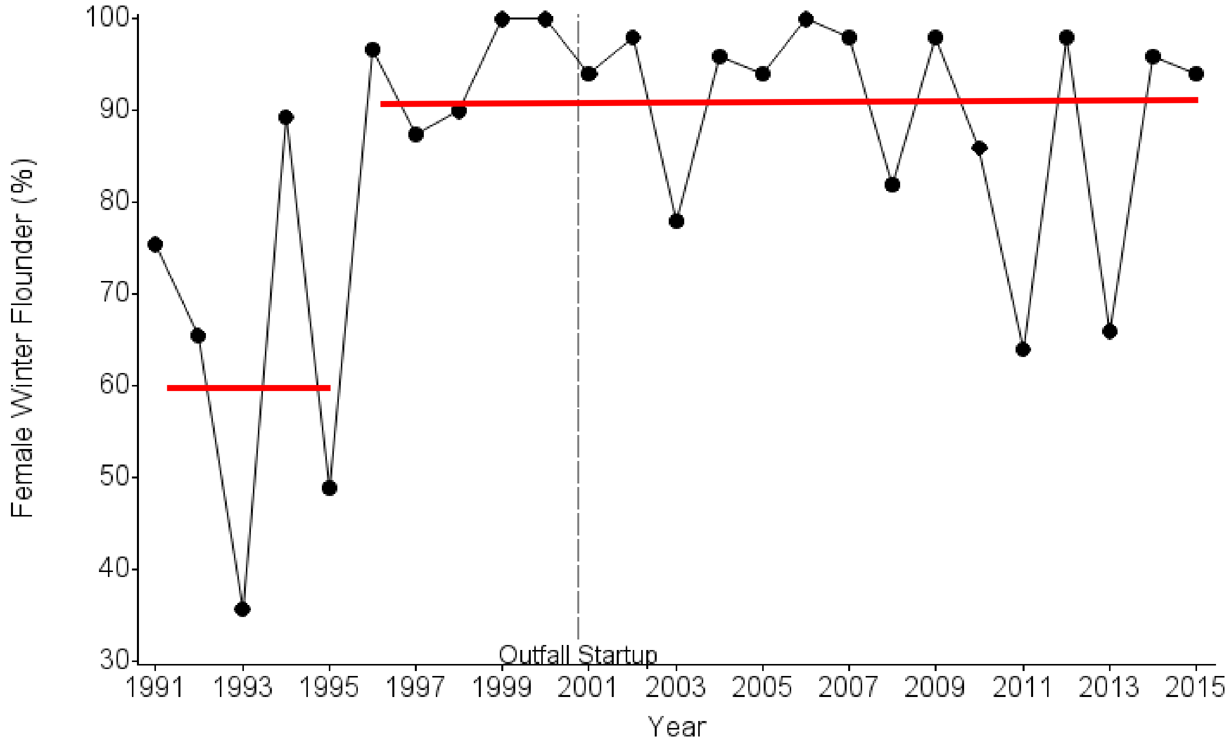

Figure 4-1. Percent Female Winter Flounder captured at Deer Island Flats, 1991-2015. Red line represents trend of time series in each segment of the time series.

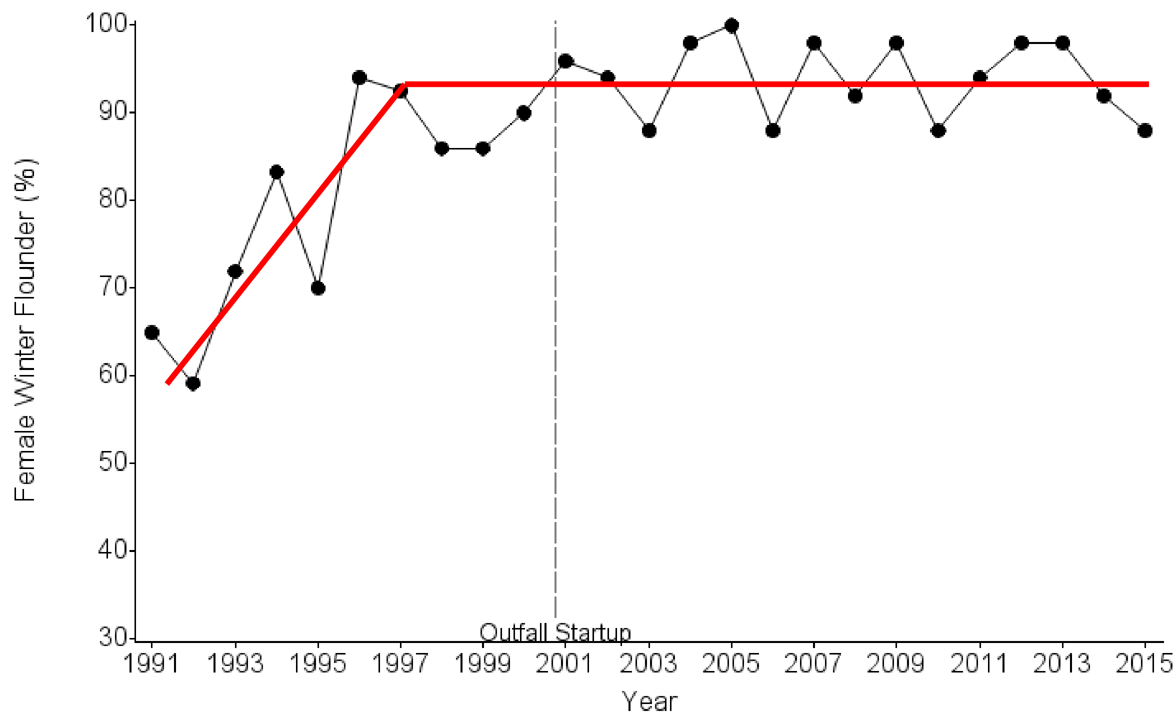

Figure 4-2. Percent Female Winter Flounder captured at East Cape Cod Bay, 1991-2015. Red line represents trend of time series in each segment of the time series.

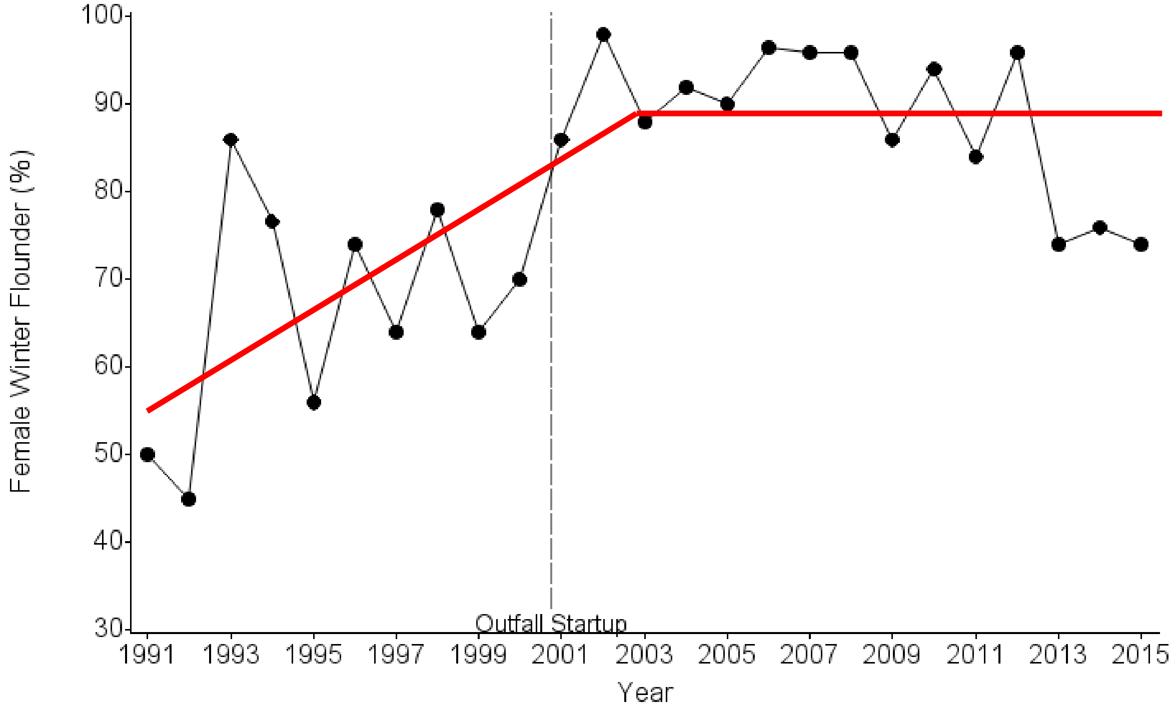

Figure 4-3. Percent Female Winter Flounder captured at Nantasket Beach, 1991-2015. Red line represents trend of time series in each segment of the time series.

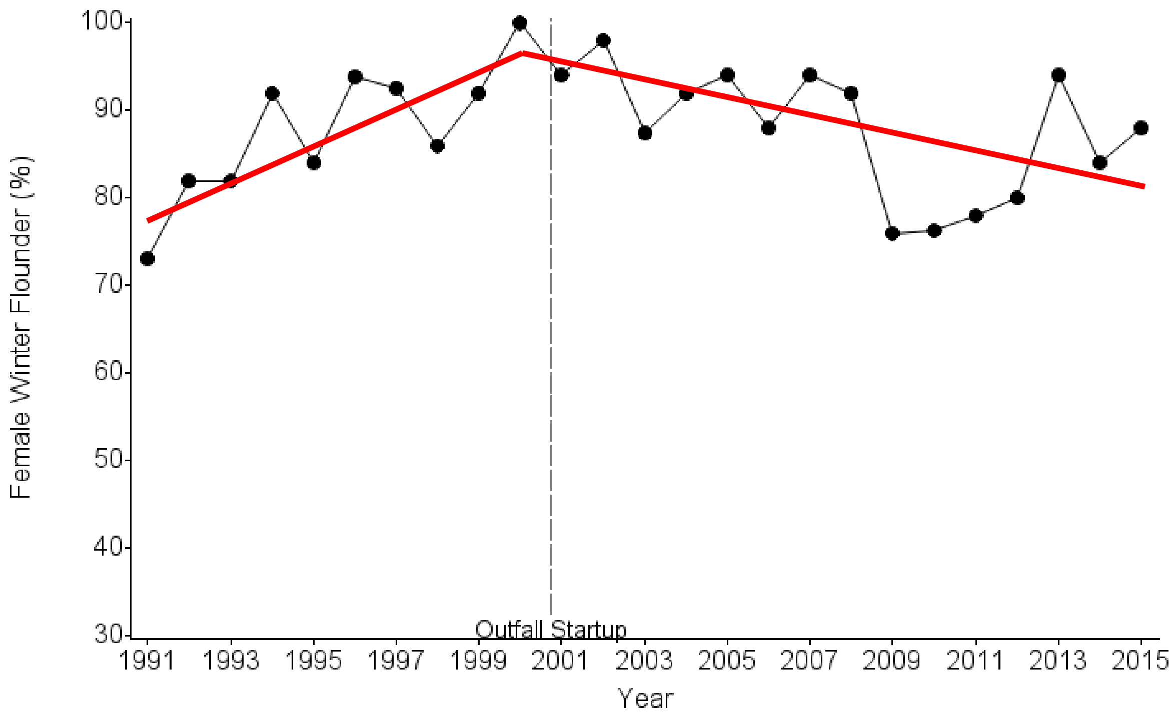

Figure 4-4. Percent Female Winter Flounder captured at Outfall Site, 1991-2015. Red line represents trend of time series in each segment of the time series.

There was a different pattern at the Outfall Site where there was a positive trend in percentage of females from 1991 through 2000, but then a negative trend from 2001 through 2015 (Table 4-1, Figure 4-4). The percentage of females was 86% in from 1991 through 2000 and 89% from 2001 through 2015.

At each station there were two segments in the time series of percentage of females. At each of the stations the percentage of females was higher in the later period compared to the earlier period, although at the Outfall site there was little difference and the percentage of females was decreasing in the later period. In addition, the percentage of females was highest at the Outfall site in 2000, one year before the offshore discharge started. All Stations except Deer Island Flats had a positive trend in an earlier segment that ended in the mid-1990s or early 2000s. There was no trend in the earlier segment at Deer Island Flats that also ended in the mid-1990s. No station exhibited an increasing trend in percentage of female in the latter segment and there was no common breakpoint in segments among stations.

Table 4-1. Trends in percentage of female Winter Flounder at MWRA sampling stations.

Station	Period	Trend	Mean Percentage Females
Deer Island Flats	1991-1995	No trend	63%
	1996-2015	No trend	91%
East Cape Cod Bay	1991-1996	Positive	74%
	1997-2015	No trend	93%
Nantasket Beach	1991-2002	Positive	71%
	2003-2015	No trend	88%
Outfall Site	1991-1999	Positive	86%
	2000-2015	Negative	89%

4.1.2 Standard Length

Standard length (SL) as measured in millimeter (mm) is the distance from the most anterior point of the fish to the end of the hypural plate (end of the last vertebra). Standard length and total length were measured in the field as part of the shipboard processing (Geoghegan *et al*. 2014). Only winter flounder greater than 300 mm total length were processed.

At Deer Island Flats there were two trends in mean SL that changed between 2001 and 2002 (Table 4-2). From 1994 through 2001 there was a positive trend in SL and the mean SL was 300 mm. From 2002 through 2015 the trend in SL was negative and mean SL was 306 mm.

East Cape Cod Bay also had a positive trend in SL that lasted from 1992 through 2005 (Table 4-2). There was no significant trend from 2006 through 2015. Mean SL was 285 mm from 1992 through 2005 and 294 mm from 2006 through 2015.

Nantasket Beach exhibited the same patterns as Deer Island Flats with a positive trend in SL from 1992 through 2007 (Table 4-2). From 2008 through 2015 there was a negative trend. Mean SL varied only slightly between segments with a mean SL of 288 mm from 1992 through 2007 and 287 mm from 2008 through 2015.

Finally, trends in SL at the Outfall Site were in agreement with the trends at the other stations (Table 4-2). SL increased from 1992 through 2005 and then decreased from 2006 through 2015. As with Nantasket Beach, mean SL differed only slightly between periods and was 287 mm in the first segment and 288 mm in the second.

To summarize the trends in SL, there was an increasing trend in SL from the early 1990s to 2005-2007 at most stations, and a decreasing trend in SL after that. Deviations to this pattern occurred at Deer Island Flats were the increasing tend ended in 2001, and at East Cape Cod Bay where there was no trend in the latter segment of the time series.

Table 4-2. Trends in mean standard length (mm) of Winter Flounder at MWRA sampling stations.

Station	Period	Trend	Mean Standard Length (mm)
Deer Island Flats	1994-2001	Positive	300
	2002-2015	Negative	306
East Cape Cod Bay	1992-2005	Positive	285
	2006-2015	No trend	294
Nantasket Beach	1992-2007	Positive	288
	2008-2015	Negative	287
Outfall Site	1992-2005	Positive	287
	2006-2015	Negative	288

4.1.3 Weight

Weight of winter flounder in grams (g) was measured in the field as part of the shipboard processing (Geoghegan *et al*. 2014).

At Deer Island Flats there was no trend in mean weight from 1993 through 1999 (Table 4-3). From 2000 through 2015 there was a negative trend. Mean weight was 518 g in the first segment and 636 g in the second.

There was a significant increase in average weight of winter flounder from East Cape Cod Bay between 1999 and 2000. There were no trends in mean weight from 1993 through 1999 or from 2000 through 2015 (Table 4-3 Mean weight was 477 g in the first segment and 539 g in the second.

Nantasket Beach exhibited the same pattern of no trend in weight in an early segment of the time series, this time from 1993 through 2001 (Table 4-3). However, weight increased substantially between 2001 and 2002 and there was a negative trend from 2002 through 2015. Mean weight was 487 g in the first segment and 547 g in the second.

The Outfall Site had a different pattern in mean weight compared to the other stations. There was a positive trend in weight from 1993 through 2006 and then a negative trend from 2007 through 2015 (Table 4-3). There was a substantial drop in mean weight between 2006 and 2007. Mean weight was 539 g in the first segment and 519 g in the second.

At all stations except the Outfall Site, there was no trend in mean weight in an earlier period that ended between 1999 and 2001 (Table 4-3). After that there were negative trends at Deer Island Flats and Nantasket Beach, and no trend at East Cape Cod Bay. The Outfall Site was different from the other stations and had a positive trend from 1993 through 2006 and a negative trend after that.

Table 4-3. Trends in mean weight (g) of Winter Flounder at MWRA sampling stations.

Station	Period	Trend	Mean Weight (g)
Deer Island Flats	1993-1999	No trend	518
	2000-2015	Negative	636
East Cape Cod Bay	1993-1999	No trend	477
	2000-2015	No trend	539
Nantasket Beach	1993-2001	No trend	487
	2002-2015	Negative	547
Outfall Site	1993-2006	Positive	539
	2007-2015	Negative	519

4.1.4 Age

Age in years was determined during most years by examination of scales in the laboratory. For 2014 and 2015, reading otoliths was the preferred technique but scales were also used if an age could not be determined from the otolith (Geoghegan *et al*. 2014). The use of otoliths as an aging method may provide an older age for larger fish than the scale method (Moore *et al*. 2014).

At Deer Island Flats there were two segments in the time series: 1991 through 2000 and 2001 through 2015 (Table 4-4). There was an increase in average age in the second period compared to the first, but neither period exhibited a trend. Mean age in the earlier segment was 4.3 years and mean age in the second segment was 4.8 years, indicating an increase in age between 2000 and 2001. The older average age in the later segment was influenced by the oldest mean ages in the time series occurring in 2007 and 2014.

At East Cape Cod Bay there was no trend in age from 1991 through 2002, and a positive trend from 2003 through 2015 (Table 4-4). Mean age was 4.1 years in the earlier segment and 4.8 years in the second.

Trends in age at Nantasket Beach were similar to Deer Island Flats. There were two segments, 1991 through 2001 and 2002 through 2015, each without trends (Table 4-4). Mean age in the first segment was 4.3 years and mean age was 4.6 years in the second. This pattern indicates a substantial increase in age between 2001 and 2002 which made a major contribution to the older mean age in the later segment of the time series.

At the Outfall Site there was one continuous positive trend in age in the time series with an average age of 4.7 years (Table 4-4). The oldest mean ages in the time series occurred in 2011, 2013, and 2014.

There were no trends in age at Deer Island Flats, East Cape Cod Bay, and Nantasket Beach during an earlier period that ended between 2000 and 2002. After that there were also no trends at Deer Island Flats and Nantasket Beach and a positive trend at East Cape Cod Bay. The Outfall Site was unusual in that there was one continuous positive trend in age throughout the time series.

Table 4-4. Trends in mean age (years) of Winter Flounder at MWRA sampling stations.

Station	Period	Trend	Mean Age (years)
Deer Island Flats	1991-2000	No trend	4.3
	2001-2015	No trend	4.8
East Cape Cod Bay	1991-2002	No trend	4.1
	2003-2015	Positive	4.8
Nantasket Beach	1991-2001	No trend	4.3
	2002-2015	No trend	4.6
Outfall Site	1991-2015	Positive	4.7

4.1.5 Catch per Unit Effort

Catch per unit effort (CPUE) of winter flounder was determined in the field as the number of fish per minute of trawling. Data were standardized as described in Section 3.1. It can serve as an indicator of abundance of winter flounder. In recent years, CPUE has also been influenced by the presence of ghost lobster traps, particularly at the Outfall Station. These traps can become tangled in the trawl reducing its effectiveness. However, any winter flounder > 300 mm TL captured in the sample are counted. Thus the presence of ghost traps in a trawl sample increases the amount of time required to collect the fish resulting in a lower CPUE. CPUE as measured in this program does not provide a good indicator of winter flounder abundance.

There was no trend in CPUE at Deer Island Flats from 1991 through 2001 and then a negative trend from 2002 through 2015 (Table 4-5). However, CPUE (0.94) was higher in the second segment compared to the first (0.32). There was a large increase in CPUE between 2001 and 2002 and a peak in CPUE that occurred in 2005 that contributed to the higher CPUE in the later segment.

Table 4-5. Trends in catch per unit effort (fish/minute of trawling) of Winter Flounder at MWRA sampling stations.

Station	Period	Trend	Catch per Unit Effort (fish/minute of trawling)
Deer Island Flats	1991-2001	No trend	0.32
	2002-2015	Negative	0.94
East Cape Cod Bay	1991-2008	No trend	0.89
	2009-2015	No trend	1.61
Nantasket Beach	1991-2002	No trend	1.00
	2003-2015	No trend	0.35
Outfall Site	1991-1999	No trend	0.61
	2000-2015	Negative	1.24

East Cape Cod Bay had no trend in CPUE during an earlier period (1991-2008) and also a later period of 2009 through 2015 (Table 4-5). CPUE was lower in the earlier segment (0.89) compared to the late period (1.61) indicating an increase in CPUE between 2008 and 2009.

Nantasket Beach was similar to Deer Island Flats in that there was no trend in CPUE during an earlier period that lasted from 1991 through 2002, and no trend in a later period of 2003 through 2015 (Table 4-5). CPUE was higher (1.00) in the earlier segment compared to the later segment (0.35). This indicates a decrease in CPUE that began between 2002 and 2003 and is reflected in the large drop in CPUE between those years.

CPUE at the Outfall Site also exhibited the pattern of no trend in an earlier segment, this time 1991 through 1999 (Table 4-5). From 2000 through 2015 there was a negative trend in CPUE. The decreasing trend in CPUE in the later segment was driven by the large decreases in CPUE from the relatively high CPUE in 2001 through 2006 and the remainder of the time series. CPUE was lower in the earlier segment (0.61) compared to the later segment (1.24), indicating an increase in CPUE that started between 1999 and 2000.

CPUE at all station except Nantasket Beach was lower in an earlier segment compared to a later segment of the time series. In addition, at all stations there were no trends in CPUE in an earlier segment of the time series. This earlier period of no trends ended in the late 1990s and early 2000s at Deer Island Flats, Nantasket Beach, and the Outfall Site. At East Cape Cod Bay the segment of no trends ended later, between 2008 and 2009.

4.2 Patterns in Coastal Waters from New York and New England

4.2.1 Data from long-term state-wide monitoring programs for inshore winter flounder in comparison to MWRA data

Sex ratio data for winter flounder were obtained from long-term coastal monitoring programs conducted from Maine to New Jersey (excluding New York) to provide perspective on sex ratio trends observed in the MWRA winter flounder monitoring program. Most of these programs have data on annual collections from at least the 1990s to present. Although analysis of sex ratio is not the principal focus of any of these programs, most of them evaluate sex in a size-stratified subset of their catch, usually in association with analysis of age and growth. The total number of flounder sexed as part of these individual programs sometimes exceeds 10,000 fish, covering a wide spatial range within their jurisdiction, thus providing a rich data set against which to compare the results of the MWRA annual sampling in Boston Harbor, and Massachusetts and Cape Cod Bays. We also obtained data from NOAA's offshore survey of the George's Bank (GB) stock, a discrete population of winter flounder that is less likely to be influenced by pollution from coastal areas in comparison to fish residing inshore. Winter flounder from GB represent a different stock and are managed separately from the Gulf of Maine (Maine down to northern edge of Cape Cod) and Southern New England/Mid Atlantic stocks (Outer Cape, southern Massachusetts down to the entrance of Chesapeake Bay) because they appear to be reproductively isolated (NEFSC 2011). Further, early work evaluating well-established biomarkers of exposure to organic contaminants (EROD activity and CYP1A content), reported the lowest values observed were from GB winter flounder. Historical analysis of winter flounder from GB collected in the late 1980s showed no evidence of contaminant related histopathology such as vacuolation or neoplasia (Moore 1991). Levels from northern Maine and Nova Scotia were also well below levels observed at all other coastal sites from New York up through Massachusetts (Monosson and Stegeman 1994). These data indicate that in the early 1990s exposure of winter flounder to coastal contamination was pervasive throughout industrialized coastal areas of the Northwest Atlantic.

Most of the state-wide surveys (ME/NH, MA, CT, and NJ coastal trawl surveys) and the NEFSC survey only determine sex for a set number of fish from each size class of fish collected (one to several fish sexed for each 1 or 2 cm size range in total length). As sex is determined on a larger percentage of larger fish (as compared to smaller ones), while this method provides a good measure of age at length, the subset of fish sexed does not represent the sex ratio for the entire population. Although the RI Department of Marine Fisheries conducts a state-wide trawl survey, sex was determined on a very limited number of fish caught, therefore the RI state data was not included in this analysis. However, the Graduate School of Oceanography at the University of Rhode Island (RI-GSO) has been conducting a long term survey, where sex is estimated on all fish caught using a visual inspection technique termed "candling" where sex is inferred from the size and shape of the gonad visible through the body of a fish held up to the light. Although the accuracy of this method is not verifiable, in most cases evaluator bias does not seem to favor one sex or another (Joe Langan pers. Com. GSO). The RI-GSO data only samples 2 locations in lower Narragansett Bay. However, to avoid having to neglect this area of the coast entirely, and because of the large number of records and long temporal coverage, we decided to include the RI-GSO data in our analyses (henceforth just referred to as RI data). New York does not conduct an annual state-wide survey of its own (although the waters of Long Island Sound which are split between NY and CT jurisdiction are sampled as part of the CT trawl survey).. During the period of 1986-1994 the NY State Department of Environmental Conservation conducted a survey of winter flounder caught by party boat fisherman (Mooney 2006). Although a significant number of fish were evaluated during this period, sex was only assessed based on scale roughness, and the sampling pattern was not random. The study was discontinued more than a decade ago, so these data were also not included in this analysis. Information on the monitoring programs that supplied the data discussed in this report is provided in Table 4-6.

Table 4-6. Summary data for surveys used in this analysis.

Area	Period	# of Records	Type of Sampling	Agency	Contact Person
New and old outfalls & reference areas	1991-2015	6,039	50 fish per site	MWRA	Ken Keay
ME/NH	2001-2015	7,193	Sex/age stratified by size	ME DMR	Sally Sherman
MA	1991-2015	15,589	Sex/age stratified by size	MA DMF	Matt Camisa
RI	1986-2015	14,440	Sex estimated visually on all fish	URI GSO	Jeremy Collie
CT	1992-2015	16,689	Sex/age stratified by size	CT DEM	Penny Howell
NJ	1993-2015	9008	Sex/age stratified by size	NJ DFW	Linda Barry
Georges Bank	1985-2015	3,921	Sex/age stratified by size	NOAA-NMFS	Paul Nitschke

There were several constraints apparent when we began evaluating these data sets to make appropriate comparisons to the MWRA data. The MWRA data is primarily collected in the spring, mostly in April and May, and because the survey was designed to monitor for liver pathology in these fishes (Moore et al. 2014), only fish of ≥30 cm total length were targeted for collection. The logic for this selectivity was that the pathology of interest was a long-term gradual development of pre-neoplastic and neoplastic lesions. Thus older larger fish are the most sensitive age and size groups to survey. There are two very important points to make concerning collecting fish of these sizes. Looking at the number of flounder sexed from all the surveys combined, the number of fish caught peaks at a ~ 30 cm total length (TL), dropping precipitously to the point where only a very small number of fish are caught of >50 cm TL (Figure 4-5a). Thus, as size increases, biases introduced by sampling a set number of fish at each size will increase, particularly at the largest sizes. The importance of size on sex ratio is clearly illustrated in Figure 4-5b, where the percent of female fish as a function of TL (again combining all the data from the state surveys as well as the MWRA data, but excluding the GB data) is shown. In this figure where only flounder > 10 cm TL (below which many of the individuals have not become sexually mature, and therefore cannot be reliably sexed), and < 50 cm TL are plotted, it can be seen that individuals of 30 cm TL fish are ~70% female, and those >40 cm TL are more than 90% female. When we plotted % female as a function of size (10-50 cm LT) for each data set except Georges Bank (Figure 4-6), it can be seen that there are big swings in sex ratio in flounder < 30cm as well flounder > 40 cm. Therefore we chose to focus the majority of our evaluation on flounder ranging in size between 30 and 40 cm TL. The size distribution of flounder from GB was distinct from all other data sets. This is apparent in Figure 4-7 where sex ratio for each site is plotted as a function of size (between 30-40 cm TL), this time including the GB fish. Within this size range, the flounder from all but one of the coastal sites (ME/NH) have a significantly positive trend in female biased sex ratio as a function of size (Table 4-7). In contrast to all other sites, there is no apparent increase in females biased sex ratio as a function of size in this range for the GB flounder which show the opposite, although not statistically significant, trend ($p = 0.055$; Table 4-7).

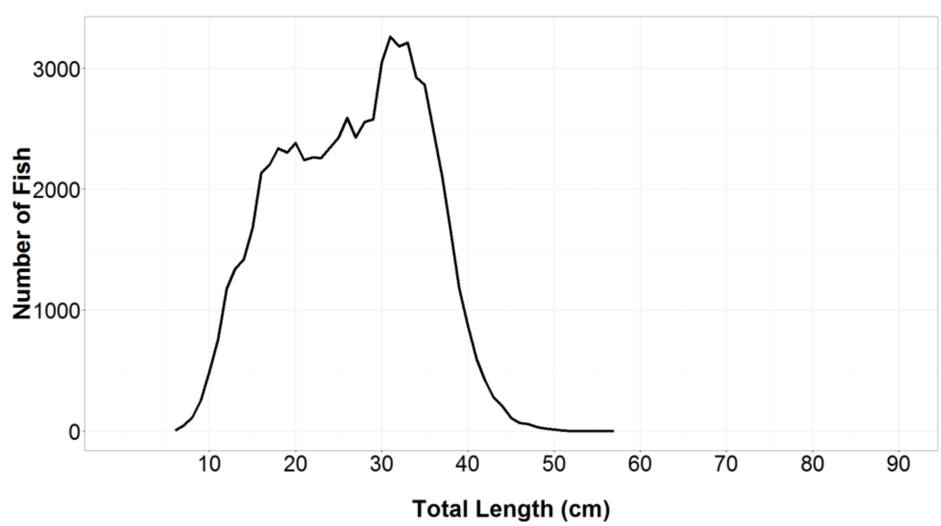

Figure 4-5a. Number of winter flounder as a function of total length (all datasets included except Georges Bank).

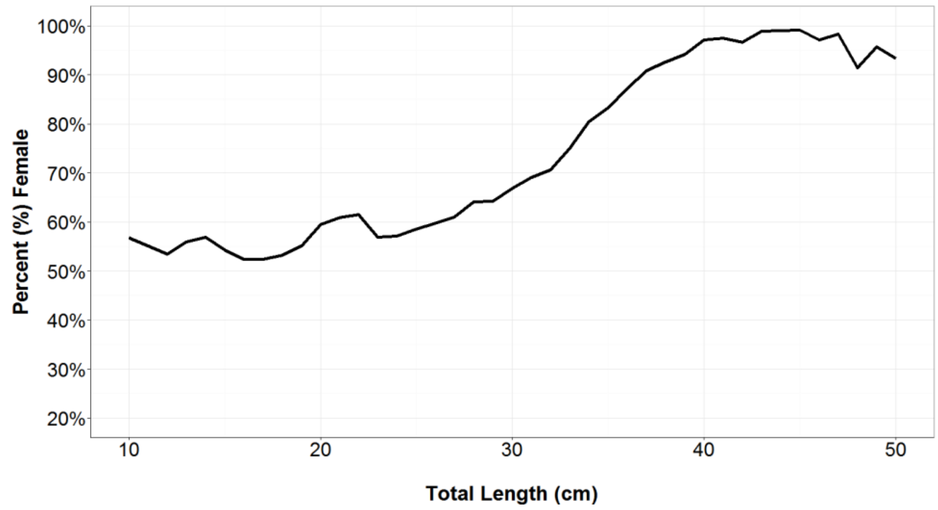

Figure 4-5b. Percent female in spring-caught flounder as a function of total length (range: 10-50 cm TL) for all data sets except Georges Bank. If abundance of males or females in a size bin was 0, 1 was added. If no fish were reported in a size bin, that bin was removed.

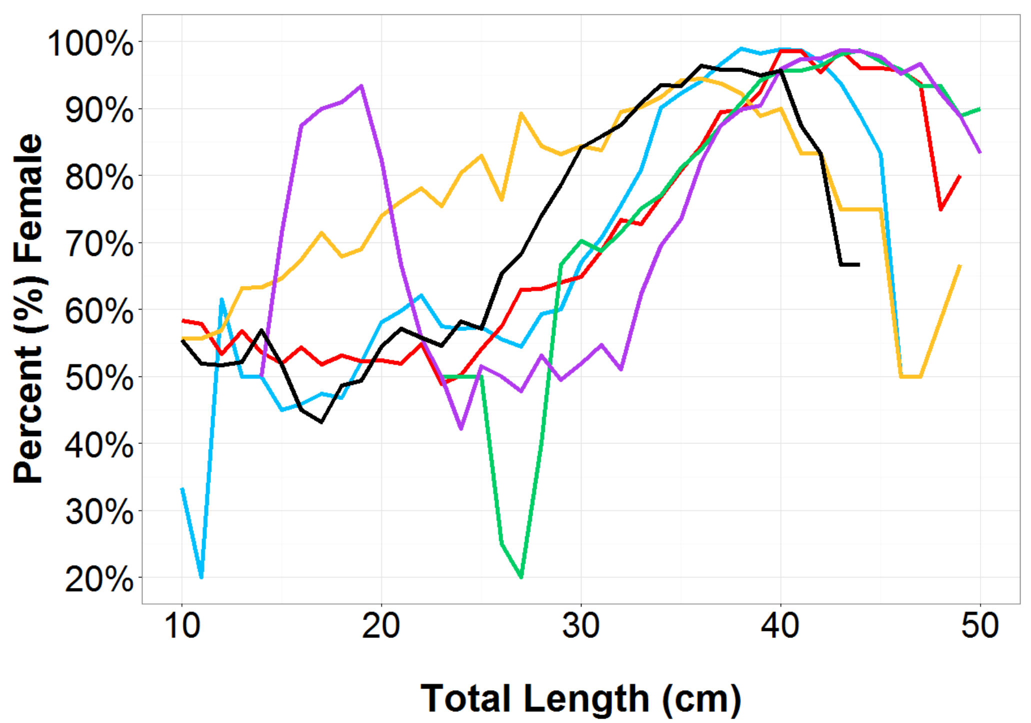

Figure 4-6. Percent female in spring-caught flounder as a function of total length (range: 10-50 cm TL) for each data set except Georges Bank. If abundance of males or females in a size bin was 0, 1 was added. If no fish were reported in a size bin, bin removed. (Purple = NJ; blue = CT; black = RI; red = MA; green = MWRA; gold = ME-NH)

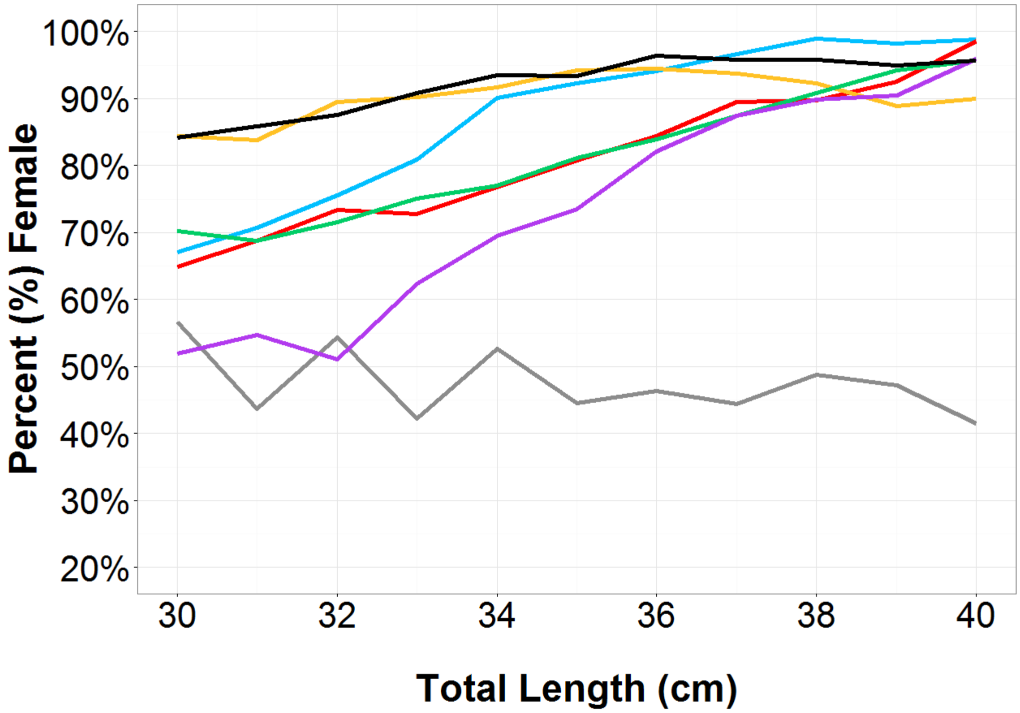

Figure 4-7. Percent female in spring-caught flounder as a function of total length (range: 30-40 cm TL) including Georges Bank. (Purple = NJ; blue = CT; black = RI; red = MA; gray = GB; green = MWRA; gold = ME-NH)

Table 4-7. Regression statistics for percent female as a function of size; data used: 1985-2015, spring fish, 30-40 cm TL.

Survey	p-value
All surveys together	<0.001*
CT	<0.001*
GB	0.055
MA	<0.001*
ME/NH	0.077
MWRA	<0.001*
NJ	<0.001*
RI	<0.001*

4.2.2 Properties of the Data Sets Compared

Abundance of near shore winter flounder is also highly dependent on season. CPUE from the south shore of Long Island changed dramatically with season from the summer when they were almost absent from the sampling area, to winter when they are relatively abundant (Keith Dunton, Monmouth University, personal communication). To avoid potential bias from seasonal use of habitat, and to make an appropriate comparison to the MWRA data which primarily samples only in spring, we also decided to only compare spring (March, April, May) data from the various sampling programs, when flounder abundance is high.

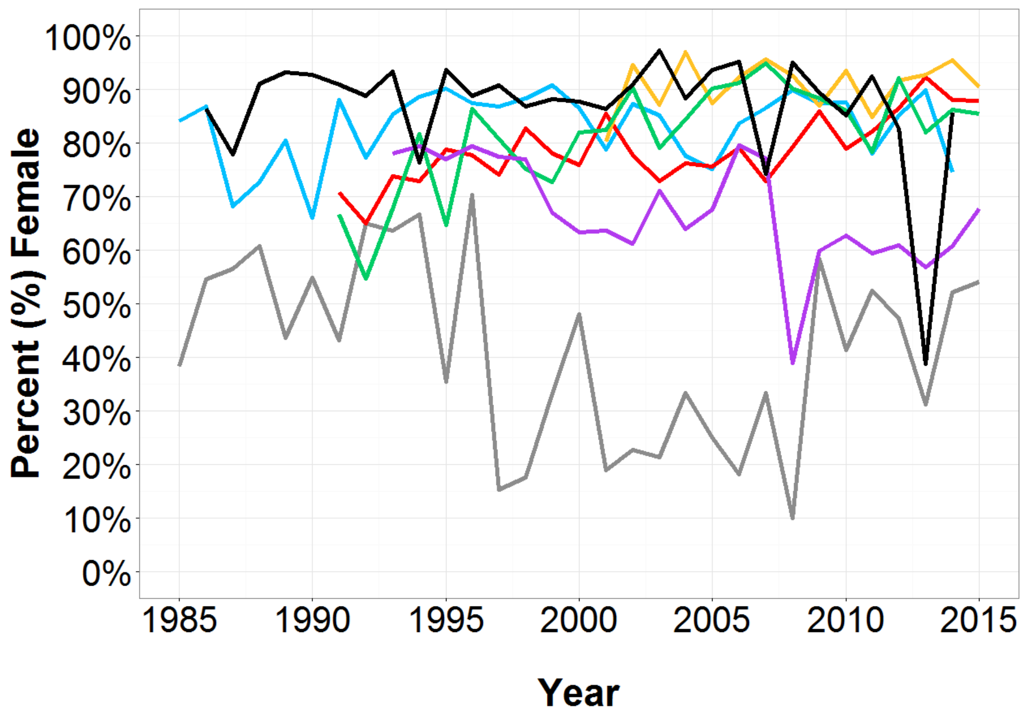

Figure 4-8. Percent female in spring-caught flounder over time (range: 30-40 cm TL). (Purple = NJ; blue = CT; black = RI; red = MA; gray = GB; green = MWRA; gold = ME-NH)

4.2.3 Temporal Trends in Sex Ratio

Data for percent female fish in spring caught flounder between 30 and 40 cm TL for all the state surveys, and the GB flounder collected as part of NOAA's NEFSC trawl survey are shown in Figure 4-8. In all data sets year to year variation is high, often varying between 10-20%, but in some cases swings of as much as 50% were observed. Thus, just looking at sex ratios for a single year may be misleading. Comparing among the data sets, the GB stock stands out as consistently having a less biased female sex ratio, even among these larger fish. Simple linear regression analysis of these data reveals statistically significant trends over time for all surveys together, as well as for the MA, MWRA, NJ, and GB data sets, while trends over time in the CT, ME/NH, and RI data sets were not (Table 4-8). Temporal trends were most significant ($p<0.001$) for the MA, and MWRA data sets which both showed positive trends with the percentage of female fish increasing over time. This is in contrast to the patterns seen in the GB and NJ flounder, where a less pronounced but statistically significant decreasing trend in the percentage of females was observed. However, looking at the time trends in the GB fish, there appears to be a biphasic response over time with percent females dropping over the period from 1985 to 2004, and then increasing from 2004 to the present. Despite the more recent increase in the percentage of females in the GB flounder population, sex ratios rarely exceed 50% in any given year. For comparison we also plotted the data for all fish >30 cm TL, to see how much of a difference excluding the fish >40 had on temporal patterns. As can be seen in Figure 4-9, the pattern is not noticeably different from that seen in the flounder of 30-40 cm TL (Figure 4-8) indicating that sexual bias contributed by the really large flounder is not a factor in the analysis.

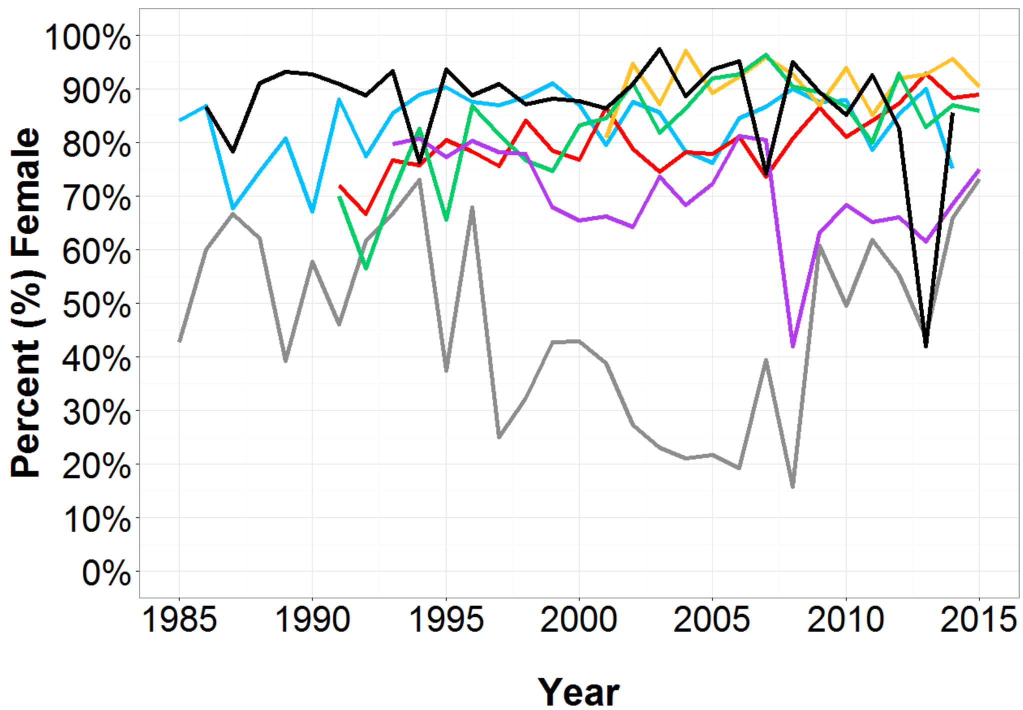

Figure 4-9. Percent female in spring-caught flounder over time (range: >30 cm TL). (Purple = NJ; blue = CT; black = RI; red = MA; gray = GB; green = MWRA; gold = ME-NH)

Table 4-8. Regression statistics for percent female over time; data used: 1985-2015, spring fish, 30-40 cm TL.

Survey	Direction	p-value
All surveys together	Positive	<0.05*
CT	None	0.244
GB	Negative	<0.05*
MA	Positive	< 0.001*
ME/NH	None	0.392
MWRA	Positive	<0.001*
NJ	Negative	<0.01*
RI	None	0.176

Table 4-9 provides the average sex ratio for the various size classes of spring caught fish from each survey and the survey's combined (with or without including the flounder from GB). The average sex ratio for all flounder is 78% female in the 30-40 cm TL, but only 66% female if fish of all sizes are considered, highlighting the influence of smaller individuals on sex ratios. In contrast, excluding the fish >40 cm TL has almost no influence on average sex ratio, indicating that there are very few individuals in this size range. It is also worth pointing out that when the GB flounder are excluded, flounder of all sizes are 67% female and those of >30 cm TL are 81% female. The site-specific averages for each site vary between 47% (GB) and 89% (MA and RI) in the 30-40 cm TL size class. Even considering the large variation observed and statistically significant trends over time in many of the data sets, significant differences among the sites in the total data set were also observed (Table 4-9), regardless of what size range of flounder were compared. Multiple range tests of the fish >30 cm TL confirmed that the sex ratio of the GB flounder were significantly different from all but the NJ fishes (data not shown).

Table 4-9. Average percent female in each survey & results of Kruskal-Wallis test [non-parametric one-way ANOVA run across surveys (CT, GB, MA, ME/NH, NJ, and RI) for each size range (all sizes, >30 cm, and 30-40 cm)].

	CT	GB	MA	ME/NH	MWRA	NJ	RI	All	All w/o GB	p-value
All sizes	68%	48%	66%	72%	83%	65%	56%	64%	67%	<0.001
>30 cm	84%	47%	80%	91%	83%	71%	87%	75%	82%	<0.001
30-40 cm	83%	43%	79%	91%	81%	67%	87%	73%	81%	<0.001

As the MA DMF survey is spatially most closely representative of the area targeted by the MWRA flounder monitoring program, we looked more closely at this data set. Figure 4-10 compares just the data from the MA DMF data for MA coastal waters north of Cape Cod (those that are considered part of the Gulf of Maine (GOM) stock) and the MWRA surveys for flounder >30 cm. The two data sets track each other very well, indicating the data observed in the MWRA survey (which samples only 4 sites), does not present a pattern demonstrably different from that apparent in the much larger survey conducted by the MA DMF.

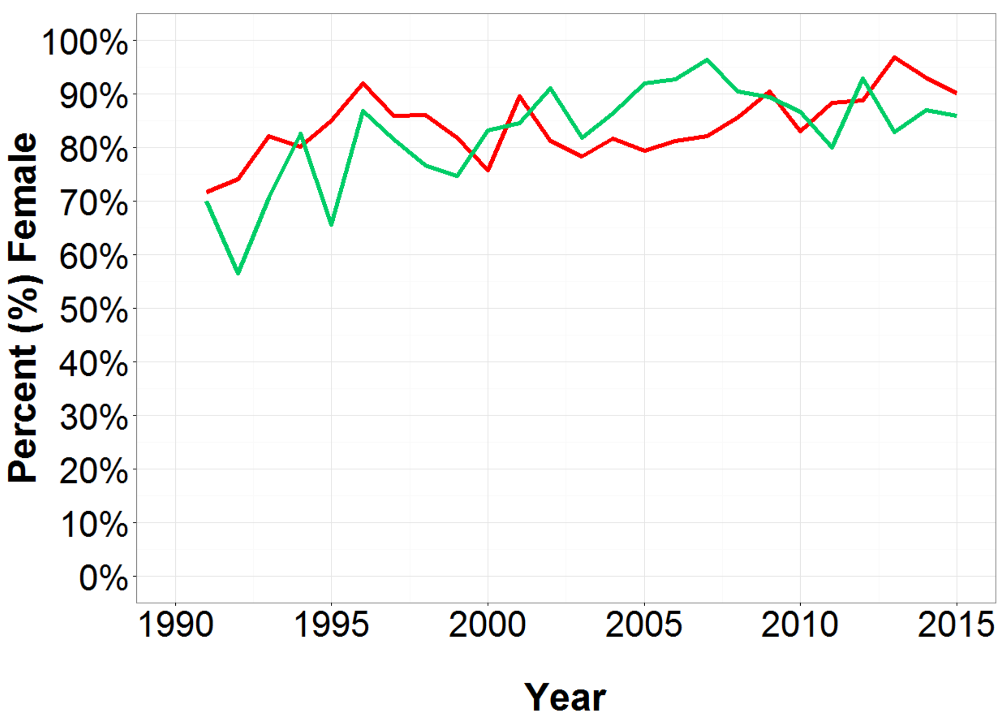

Figure 4-10. Percent female in spring-caught flounder over time (range: >30 cm TL): comparing MA (Mass-DMF, just Gulf of Maine stations) and MWRA data sets. (Red = MA-GOM; green = MWRA)

4.3 Discussion

There are many environmental factors that can influence sex ratios in fish; chief among them are temperature and exposure to chemicals that can act to mimic or block the action of endogenous hormones (see review by Lukenback et al. 2009). For species, or even populations, showing temperature dependent sex (TDS) determination, environmental temperatures during a thermosensitive window (usually during early development and/or sexual maturation) can lead to highly biased female or male dominated populations. TDS has been observed in a number of flatfish species (Lukenback et al 2009). While it has not been shown for winter flounder, data from a laboratory study conducted on winter flounder by Elizabeth Fairchild (Fairchild et al. 2007) has raised the question of whether or not TDS may have been a factor in their results. There is evidence from many laboratory and field studies demonstrating the potential for environmental chemicals (natural and synthetically produced hormones and chemicals that mimic or inhibit them) to either feminize or masculinize fish, occasionally leading to reduced fertility or even infertility with the potential for population level consequences (Sumpter and Johnson 2006). Given the depressed state of inshore winter flounder stocks in all but ME/NH (ASMFC 2014), it is very important to understand any negative influences of anthropogenic activities on the recovery of these stocks.

Concern about the potential influence of estrogenic chemicals (natural and synthetic hormones, and other hormonally active chemicals) introduced by sewage effluent is based on the seminal work of John Sumpter's group in England which demonstrated wide spread feminization in both freshwater and marine fishes exposed to sewage effluent or from highly urbanized coastal waters (reviewed in Matthiessen et al. 1998). In their study of coastal flatfish (European flounder, *Platicthys flesus*), it is important to point out that even while they observed highly elevated levels of vitellogenin (VTG - a biomarker indicating exposure to estrogenic compounds) in wild caught fish, almost no evidence of ovotestes (a condition where male gonads contain oocytes) was observed, and most importantly, no evidence of female biased sex ratios were reported. They also failed to observe correlations between VTG induction and volume of domestic sewage discharged to coastal waters (Matthiessen et al. 1998). Feminization (increased VTG levels) has been observed more locally in juvenile (young-of-the-year YOY) winter flounder from urbanized areas in New York Coastal waters (McElroy et al., 2006 and 2015), but a detailed analysis of altered sex ratios in wild caught fish has not, to our knowledge, been conducted in YOY winter flounder. Duffy et al. (2009) did find a positive correlation between female biased sex ratios and an urban gradient in a large three-year survey of adult silversides (*Menidia menidia*), a relatively short lived species, conducted around Long Island, NY. Therefore when an increasing female bias in the sex ratio for flounder collected as part of the MWRA monitoring program began to be apparent, some were concerned that there might be a link to sewage derived compounds either associated with contaminated sediments from the old outfall at Deer Island Flats or the current outfall in Massachusetts Bay.

Even without comparing the MWRA data to data collected by the other state-wide monitoring programs, a link between the increasing trend in female biased sex ratios and sewage derived contaminants being released from the Boston outfalls (either old or new) would have been unlikely as feminization was observed at all four of the MWRA monitoring sites, including their reference sites off Nantasket Beach and Eastern Cape Cod Bay, unless there was almost perfect mixing of flounder throughout the area. Such mixing is highly unlikely given the persistent, stable differences in liver pathology prevalences recorded between the different MWRA stations (Moore et al. 2014). Such differences would not be present if the fish mixed through the study area. As shown in this report, when flounder of similar sizes are compared, the MWRA data tracks that observed throughout MA state waters as monitored by the MA DMF.

High percentages of female winter flounder have been reported before from a variety of both large and small surveys conducted within this region (from the Gulf of Maine to NJ) (Table 4-10; Fairchild 2012). The MWRA dataset is no exception to this pattern, but percentages of female winter flounder reported by MWRA are higher than reported by other researchers. The occurrence of skewed sex ratios from many locations and from at least as early as the 1950s is an indication that the high percentages of female winter flounder in the MWRA monitoring program is not a unique occurrence.

Table 4-10. Percentage of female Winter Flounder reported at locations along the east coast (adapted from Fairchild 2012, Table 2).

Location	Percentage Female	Months	Year	Reference
Ipswich Bay	88%	Feb.-Jun	2007	Fairchild *et al*. 2007
South of Cape Cod	70%	Dec.-April	1960-1965	Howe and Coates 1975
South and east of Cape Cod	56%	May	1983-1991	Witherell and Burnett 1993
Rhode Island Sound	70%	Nov.-April	1956-1958	Saila 1961
Narragansett Bay	60%	Feb.-March	1958	Saila 1962
Niantic River, CT	68%	Feb.-April	1977-2010	DNC 2011
Raritan Bay and Sandy Hook, NJ	73%	March-Sep.	1989	Bejda and Phelan 1998
Navesink Estuary	60%	Feb.-April	1997	Stoner *et al*. 1999
New York Bight	64%	Jan.-Oct.	2007	Wuenschel et al. 2009

Although statistically significant trends in female biased sex ratios were observed in the MWRA and MA DMF datasets, this was not universally observed among the different monitoring programs. The most northern data set evaluated, ME/NH failed to show a significant trend in sex ratio with time. The ME/NH data set was much shorter (data only began in 2001), but also reported a very high percentage of female flounder (91%) in spring caught fish of the size range being compared (30-40 cm TL). Neither the CT nor the RI data sets showed a significant trend in sex ratio with time, although the mean percent females (83% and 87%, respectively) in these surveys was close to those observed in Massachusetts waters by MA DMF (79%) and MWRA (81%). The NJ survey data showed a statistically significant trend in the opposite direction, as did the GB data. Despite the large year-to-year variations in sex ratio in all datasets, a simple non-parametric ANOVA detected significant differences in average sex ratio between the sites regardless of which size class of flounder were analyzed (Table 4-9). Averaged over the whole record, sex ratios were similar among all sites except GB. As mentioned earlier in this report, GB represents a different population with altered age and size frequencies, as well as other population demographics (NEFSC 2012). One could argue that the clear difference between sex ratio over time (Figures 4-8 and 4-9), and sex ratio as a function of size (Figure 4-7) observed in the GB flounder as compared to data obtained on coastal flounder is consistent with the influence of either pervasive coastal contamination, or environmental conditions in more inshore waters. However, the similarity in sex ratios observed in state surveys of inshore waters from northern ME down to CT does not really support either hypothesis, as temperature and likely chemical contamination vary substantially over this area. Several studies of winter flounder population genetics have demonstrated localized subpopulations over fairly small spatial scales (Crivello et al. 2004; Buckley et al. 2006; O'Leary et al. 2013), while one other has not (Wirgin et al.

2014), on balance indicating the absence of widespread mixing among individual flounder from all these locations.

The segmented regression analysis of the MWRA data described in Section 4.1 provides further evidence that material discharged from either the old outfall at Deer Island Flats or the new outfall are not associated with alterations in sex ratio. Despite average sex ratios being more female biased in the more recent record as compared to the earlier times points at all sites, the break points identified by this analysis failed to link any changes in sex ratio leading to increased feminization with the timing of the outfall opening. Further, although a statistically significant break point in trends, was identified at all four MWRA collection sites, the timing of the break was not consistent between site, varying between 1996 and 2003 (Table 4-1). It is interesting to note that for all four stations there was a significant trend of increasing length of the MWRA flounder during the early years when sex ratio was increasing most dramatically (Table 4-2), consistent with the hypothesis that the increase in female biased sex ratios was due to the presence of a greater number of larger fish sampled at these sites. There are many population level factors that can influence size (and age) of fishes, including winter flounder (Kindsvater et al. 2016), and due to the strong influence of size on sex ratio, population dynamics are likely to be the primary, or at least a key determinant influencing the patterns observed here.

When data collected during the same season (spring) on flounder of similar sizes (30-40 cm TL) are compared, there are no differences between the MWRA and the MA DMF surveys, indicating not only is the pattern for increasing female biased sex ratios observed at the MWRA sites a state-wide phenomenon, but that there are no differences between sites with more oceanographic connectivity to Boston Harbor and both the old and new outfall sites, waters that are home to the GOM (Gulf of Maine) flounder population, and those to the south and east of Cape Cod, home to the SNE (Southern New England) population, at least within Massachusetts state waters.

Collectively these data do not support a contaminant-linked association with sex ratio, at least not one strictly associated with sewage releases into Boston Harbor and MA Bay. Indeed if there were a significant association between contaminant inputs and sex ratio in adult winter founder from Boston Harbor, one might have expected there to be discernable difference in sex ratio between the sites, and years (in context with documented reduction of sediment contaminant levels associated with of treatment upgrades) sampled as part of the MWRA monitoring program (Hunt et al. 2006). Although there are clearly differences in sex ratio over time (evaluated more fully in the entire dataset in Section 4.2 of this report), there is no indication of differences in sex ratios associated with the four sites sampled, nor changes consistent with treatment upgrades.

5. CONCLUSIONS

The 2015 Flounder Survey provided samples from four locations (DIF, NB, OS, and ECCB) and was conducted in a manner consistent with previous surveys. Catch per unit effort at OS was close to median for that station. The overall size of the flounder collected increased during the past decade until 2008, when size returned to levels seen at the beginning of the study, a trend that continued through 2015. As has been the case throughout the duration of the monitoring program, the 2015 catches were dominated by females. Factors influencing sex ratios are complex and poorly understood. The already high proportion of females increased at all sites during the baseline period, and since the Outfall came on line the fish collected from eastern Cape Cod Bay, the site farthest from the Outfall, have had the highest proportion of female fish, until 2014 and 2015, when there were more females at DIF.

Following increased ulcer prevalence beginning in 2003, extensive pathology and microbiology studies were unable to determine a cause of the ulcers (Moore et al. 2004). Elevated levels of ulcers were observed from 2003 to 2006 at stations except for ECCB. Ulcer prevalence then decreased and remained low from 2007 to 2010, followed by an increase reported in 2011. In 2012 low levels of ulcers were present at OS and NB. No ulcers were observed in 2013, and two were observed in 2014. Ulcers were marginally more prevalent in 2015

Results of the histological analyses in 2015 support previous observations made from this long-term dataset.

- Age-corrected hydropic vacuolation prevalence data suggest that there has generally been a steady reduction in the contaminant-associated pathology in winter flounder collected at Deer Island Flats during the past two decades, although there was a marked increase at this station in 2015, returning to levels seen soon after the activation of the outfall in 2000.

- The DIF data available from the 1980's (Moore et al. 1996) were not age-corrected. Uncorrected CHV prevalences in harbor flounder have decreased from over 75% in 1988 to approximately 32% or less in recent years. This is a remarkable change.

- Flounder collected off Nantasket Beach and in the vicinity of the outfall since discharge began in September 2000 also consistently show hydropic vacuolation prevalence at or lower than levels observed during baseline monitoring (1991-2000).

- The high neoplasm prevalence characteristic of fish from DIF in the mid- to late-1980s (Moore et al. 1996) has disappeared. Neoplasia has not been observed in a fish from Boston Harbor since 2004, and has never been observed in fish collected at the outfall site.

- Disease prevalence at the Eastern Cape Cod Bay reference site has been relatively stable since monitoring began there in 1991 and is consistently the lowest of all areas sampled.

- One possible trend that will continue to be tracked is a slow rise in the prevalence of age-corrected CHV in flounder collected in the vicinity of the outfall that was observed between 2005 and 2010. CHV declined at the OS site in 2011 and 2012, rose somewhat in 2013, fell again in

2014 and raised slightly in 2015 (Figure 3-12). These values are still below baseline levels, prior to outfall startup.

- Collectively the sex ratio data from the MWRA program and from coastal New York and New England do not support a link with sewage releases into Boston Harbor and MA Bay.

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Massachusetts Water Resources Authority
Charlestown Navy Yard
100 First Avenue
Boston, MA 02129
(617) 242-6000
www.mwra.com