



Effects of Wastewater Effluent on the Sex Composition of Winter Flounder in Massachusetts Bay

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Effects of Wastewater Effluent on the Sex Composition of
Winter Flounder in Massachusetts Bay

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A Thesis in the Field of Sustainability
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

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Abstract

Wastewater treatment plant effluent enters marine and freshwater ecosystems, introducing pharmaceuticals into bays, estuaries, and rivers. It is important to understand how pharmaceuticals may be of concern when they settle into these sensitive systems. When the Massachusetts Water Resources Authority (MWRA) first began talks in the early 1990s about moving the wastewater outfall site from Boston Harbor to its present location in Massachusetts Bay, there were concerns raised about negative effects to fish and other marine species. This is true especially since the outfall is surrounded by many eco-diverse regions like Stellwagen Bank National Marine Sanctuary, Cape Cod Bay Ocean Sanctuary and North and South Essex Ocean Sanctuaries.

MWRA yearly monitoring results reveal an increasingly greater proportion of female winter flounder in Massachusetts Bay. One of their sampling locations furthest from the outfall is yielding some of the highest rates of female winter flounder, suggesting that the outfall may not be the cause. Are pharmaceuticals and personal care products (PPCPs) in effluent, or more specifically active pharmaceutical ingredients (APIs), causing the declining sex ratio? Or are other more natural factors, such as changes in water temperature, contributing to the problem?

APIs enter wastewater systems through several means, but most commonly through urine and feces from individuals taking medications. Other sources are also responsible for contributing to the problem, such as from hospitals, improper disposal of medication into toilets and sinks, as well as agricultural runoff (i.e. antibiotics from farm

animals). APIs are currently not tested at sampling locations by any of these agencies. In order to understand possible population effects from APIs in effluent, it is important to examine the geographic locations of individual fish (from the sampling data) in relation to their sex and distance from the outfall.

This research asked the question: how is winter flounder sex ratio composition tied to proximity to the Deer Island Wastewater Treatment Plant (DIWWTP) outfall site in Massachusetts Bay? The research tested the hypothesis that distance from the effluent outfall predicts sex ratio variation. Sampling data on the Gulf of Maine (GOM) winter flounder (*Pseudopleuronectes americanus*) is available through the National Marine Fisheries Service/ Northeast Fisheries Science Center (NMFS NEFSC), Massachusetts Division of Marine Fisheries (DMF), and MWRA. Data from these agencies provide thousands of GPS coordinates on the species and their corresponding biological data. Sex and maturity are captured during NEFSC and DMF sampling and provide important details on the health and future outlook for the GOM winter flounder stock.

Results revealed that sample points <10 miles and 10-20 miles from the outfall have experienced a significant increase in the proportion of female flounder. NEFSC and DMF winter flounder in size groups 29 cm and 30 cm show a 13% increase in females from the baseline (1980-2000) to the post-diversion periods (2001-2017). In order to control possible risk factors in the future, state and federal policies should include requirements on testing pharmaceutical compounds in drinking water and wastewater. Currently, the EPA does not regulate concentrations of pharmaceuticals in drinking or waste water. Policies should be enacted and enforced before irreversible damage is caused to marine and freshwater ecosystems.

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Definition of Terms

APIs: Active pharmaceutical ingredients, biologically active component of drug product

DIWWTP or DITP: Deer Island Wastewater Treatment Plant

DMF: Massachusetts Division of Marine Fisheries

Fecundity: Fertility, reproductive rate, or the ability to produce an abundance of offspring

GB: Georges Bank winter flounder stock

GOM: Gulf of Maine winter flounder stock

SNE/MA: Southern New England/ Mid-Atlantic winter flounder stock

SSB: Spawning stock biomass

MSY: Maximum Sustainable Yield

MWRA: Massachusetts Water Resources Authority

NMFS NEFSC: National Marine Fisheries Service/Northeast Fisheries Science Center
(science and research for NOAA fisheries)

NOAA: National Oceanic and Atmospheric Administration

Oocytes: An immature female egg cell in the ovary

Otolith: Each of the three small oval calcareous bodies in the inner ear of vertebrates

PPCPs: Pharmaceuticals and Personal Care Products

Vitellogenin (VTG): Yolk precursor protein

WWTP: Wastewater treatment plant

Chapter 1

Introduction

The annual decrease in commercial landings of Gulf of Maine (GOM) winter flounder, *Pseudopleuronectes americanus*, is concerning for fisherman and consumers alike (National Oceanic and Atmospheric Administration [NOAA], n.d.a). The commercial landings for winter flounder are at a historical low, but overfishing is currently not the reason for these low numbers. Flatfishes such as winter flounder are an integral part of benthic communities. Their diet mainly consists of benthic organisms such as amphipods and polychaetes, and flounder provide an important food source for higher predators, including humans (Gibson, 2005). If the population continues to decline, it will lead to local extinction (Maine.gov, 2016). Questions need to be raised when overfishing is not occurring but stocks remain low or continue to decrease. Pharmaceuticals in wastewater may be altering the natural balance in the marine ecosystem, and potentially be responsible for declining populations of flatfishes.

Active pharmaceutical ingredients (APIs) are currently released with effluent from the Deer Island Wastewater Treatment Plant (DIWWTP) outfall site in Boston Harbor, and may contribute to low seafood landings. In freshwater systems around the world, male fish show evidence of feminization, with estrogens from birth control medications being the likely cause (Niemuth & Klaper, 2015). Metformin, a diabetes medication, is one of the most commonly found pharmaceuticals in wastewater effluent and acts similarly to estrogen, causing the development of intersex gonads as well as a

reduction in fecundity in treated fish (Niemuth & Klaper, 2015). Antidepressants may also alter normal behavior, making fish avoid interactions with other fish, and even interfering with spawning. A high concentration of antidepressants was discovered in the brains, livers, muscles and gonads of fish in the Great Lakes (Arnnok, Singh, Burakham, Perez-Fuentetaja, & Aga, 2017). These various compounds are introduced into lakes, streams, rivers and oceans through wastewater treatment plants.

Without proper regulations and the removal of pharmaceuticals and personal care products (PPCPs) from wastewater effluent, these compounds will continue to enter various bodies of water. In a two-year study, 54 PPCPs and hormones were assessed from surface waters and sediment samples about two miles from a wastewater treatment plant in the Great Lakes (Blair, Crago, Hedman, & Klaper, 2013). Over 30 PPCPs were detected in the sediment, even as far as two miles from the shoreline, and included triclosan and metformin. Twenty-four compounds were detected at levels of medium to high risk, posing a significant threat to the Great Lakes.

Might APIs in wastewater effluent help explain why GOM winter flounder commercial landings are at a historical low, even though overfishing is not occurring?

Research Significance and Objectives

This research attempted to understand if the release of APIs from wastewater effluent into aquatic ecosystems may have an effect on the declining sex ratio of winter flounder. This may also be relevant to understanding the reason for decreasing commercial landings of winter flounder. This species is an important food source, and its demise will likely affect the marine ecosystem, including predators that generally prey on

them such as the cod, as well as other marine organisms that are the flounder's main food source (Gibson, 2005). In addition to understanding this delicate balance, studies are necessary to discover how consuming fish exposed to pharmaceuticals may affect human health.

The objectives of my research were to:

1. Examine how GOM winter flounder sex ratio varies with distance from the effluent outflow point; increased distance presumably correlates with lower concentrations of effluent chemicals, such as pharmaceuticals.
2. Examine winter flounder spawning locations and female fecundity to better understand possible effluent chemical exposure in those areas during early stages of development.
3. Utilize QGIS to plot thousands of GPS individual winter flounder locations obtained from the National Marine Fisheries Service/ Northeast Fisheries Science Center (NMFS NEFSC) and Massachusetts Division of Marine Fisheries (DMF).
Massachusetts Water Resources Agency (MWRA) data was used for comparison.

Background

A typical sewage treatment plant releases wastewater effluent containing nutrient and chemical contaminants into aquatic ecosystems, altering the natural balance in those environments. Active pharmaceutical ingredients (APIs) enter wastewater from human excretion, and end up in various surface waters (Kostich, Batt, & Lazorchak, 2014). In a study of 25 drinking water treatment plants across the U.S., samples were analyzed for 247 contaminants, most of which are not regulated by the US Environmental Protection

Agency in drinking or ambient water supplies (Benson et al., 2017). The results in that study revealed 41 contaminants of emerging concern (CECs) that were detected one or more times in treated drinking waters at high concentrations, with some of these contaminants posing possible public health concerns. Currently, the EPA does not have set guidelines for pharmaceuticals in drinking water or wastewater, although APIs have been appearing in samples for many years (Kostich et al., 2014). Pharmaceutical and personal care products (PPCPs) need further study and regulations as they are increasingly appearing in drinking water samples.

In a study of 50 large municipal wastewater treatment plants in the US, 56 active pharmaceutical ingredients were measured, with 90% of the samples detecting metoprolol, atenolol, and carbamazepine (Kostich et al., 2014). The highest concentration measured was for valsartan at 5300 ng/L, which also had the highest average concentration at 1600 ng/L and occurred in all 50 samples. Fluoxetine, an antidepressant, had a maximum value of 31 ng/L. Although risk to humans may be low, there could be risks to aquatic life exposed to these compounds (Kostich et al., 2014). Fluoxetine altered normal mating behavior in a study on fathead minnows, with more profound impacts to the male population (Weinberger & Klaper, 2014). This was true at low or high levels of fluoxetine, where male aggression and predator avoidance were observed, and female egg production and feeding were affected. The reproductive health of male smallmouth bass in the Washington, D.C. and Maryland portions of the Potomac watershed were affected by their proximity to wastewater treatment plant effluent, with a high prevalence of testicular oocytes in males, and decreased circulating vitellogenin (VTG) in females (Iwanowicz et al., 2009). The study also mentions non-point sources, such as agricultural

and urban runoff, contributing to these reproductive issues. In the Weweantic River in Massachusetts, female winter flounder tissues showed high concentrations of pesticides, with highest concentrations in ripening ovaries (Smith & Cole, 1970).

Estrogen causes increases in metabolic activity in the liver, and an increase in the amount of VTG in sexually mature males (Emmersen & Emmersen, 1976). Some of the more concerning compounds found in effluent include estrogens, which can affect the endocrine glands in exposed organisms (Cavallin et al., 2016). This was observed in male and female fathead minnows that were exposed to 100% effluent containing estrogen compounds in a 21-day study. Fecundity increased when exposed to only 20% effluent, resulting in a greater cumulative mean number of eggs spawned than the control group. The 20% amount is the approximate dilution factor in Lake Superior, the area of study. Adult fathead minnows exposed to 100% effluent had reduced fecundity and spawning compared to those that were in the control group. The 100% effluent treatment caused a cessation of spawning during the first week, while yielding higher production in 20% effluent (Cavallin et al., 2016). In order for the EPA to regulate these APIs in wastewater, more studies need to be performed to understand possible risks to humans and to marine life.

Winter Flounder (*Pseudopleuronectes americanus*)

Winter flounder is mainly found in coastal areas of the Northwest Atlantic, anywhere from the Canadian province of Newfoundland and Labrador to the Southern parts of the U.S. in Georgia (Gibson, 2005). Flounder feed during the day, due to a dependence on light, and prey on small invertebrates, shrimp, clams and worms. The

name comes from the time of year it migrates inshore to estuaries for spawning. Tagging studies reveal that winter flounder generally return to the same spawning locations (Atlantic States Marine Fisheries Commission [ASMFC], 2012). Larvae and juvenile winter flounder grow and survive depending on temperature, water quality, dissolved oxygen, salinity and food availability, spending their first year of life in estuaries (NOAA, n.d.b; ASMFC, n.d.; Pearcy, 1962). Their typical habitat includes muddy sand with patches of eelgrass, but they have also been observed on cleaner sand, clay and coarser benthic substrates (Bigelow, 2002).



Figure 1. Winter flounder resting on eelgrass (NOAA, n.d.b).

Winter flounder is unmistakable due to its unusual look. It has a thick body, a straight lateral dark line, and the underside is white with both eyes located on the right

side of its body (NOAA, n.d.b). Very early in its life, the left eye migrates to the right, as the fish adapts to travelling along the bottom of the ocean. This allows the flounder to rest on the ocean bottom and look up with both eyes (Figure 1). Winter flounder bury themselves in benthic sediment, or in eelgrass, to avoid ocean currents and predators. They camouflage themselves against their background, making it difficult for both predators and prey to distinguish them from the sediment (Gibson, 2005).

Winter flounder is the only flatfish species in the northwest Atlantic that is known to spawn demersal eggs (Gibson, 2005). Unlike the majority of flatfish species that spawn pelagic eggs, or eggs which float freely within the water column, winter flounder spawn demersal eggs that sink to the bottom of the seafloor. This makes the eggs' survivability highly dependent on environmental conditions in the benthic zone, including sediment quality.

Contaminated sediments, in addition to any environmental contaminants in the water, may be absorbed directly by flounder or through feeding mainly on benthic organisms. The seafloor absorbs contaminants, increasing the concentration of these contaminants in winter flounder that bury themselves in the sediment or rest on the surface of the seafloor (Gibson, 2005).

Sediment quality, from previous or current sources of contamination, affects the survivability of the winter flounder population. Fish from contaminated areas have a higher chance of producing eggs with abnormalities, as was observed in winter flounder in Long Island Sound and Boston Harbor (Perry, Hughes, & Hebert, 1991). Contaminants were causing reproduction issues in these fish and abnormalities in developing embryos, including chromosome damage.

Maturity at Size and Growth Patterns

Winter flounder males tend to mature earlier but grow to a smaller maximum size, while females mature later but grow bigger (ASMFC, 2012). In addition to being longer, sexually mature flatfish weigh more (Emmersen & Emmersen, 1976). It is usually possible to visually differentiate sex because of the different shapes of the testis and ovary in both immature/ non-reproductive and reproductive fishes (Burton, 1994). Ripe females have distended ovaries along the entire ventral side of the fish during spawning season, and males extrude milt when pressure is applied gently to the abdomen (Fairchild, 2010). A nutritionally sensitive time for reproductive fish is prior to and following the current spawning period (Burton, 1994).

During a 1983-1991 DMF winter flounder sampling north of Cape Cod, it was observed that females matured at a length of around 28.7 cm, while males matured at 27.2 cm, or at an age of 3.3 years for both sexes (Witherell & Burnett, 1993). Sampling was conducted at 95 stations every year, with stations chosen at random. Sex was assigned based on examination of the gonads. North of Cape Cod, observations revealed that females grow longer, while south of Cape Cod the numbers are reversed with males growing longer (O'Brien, Burnett, & Mayo, 1993).

There are three federally managed stocks of winter flounder in the United States: Georges Bank (GBK), Gulf of Maine (GOM), and Southern New England/ Mid-Atlantic (SNE/MA) (ASMFC, n.d.). Of these three stocks, GBK spawn offshore for their entire life, while the others spawn inshore and move into deeper waters post-spawning (Fissures, 2017). The average size for mature GOM winter flounder males is around 27 cm, and 29.7 cm for females (P. Nitschke, National Marine Fisheries Services, personal

communication). Unlike the GOM stock, GB winter flounder mature at median lengths of 24.9 cm for females and 25.6 cm for males (O'Brien et al., 1993). Coastal stocks of winter flounder, such as GOM, are smaller for each particular age group compared to those further away like the GB stock (Witherell & Burnett, 1993). Based on known growth patterns, size is a more dependable way of assigning maturity, rather than age, for specific stocks of winter flounder (McBride, Wuenschel, Nitschke, Thornton, & King, 2013; O'Brien et al., 1993). Females grow faster and larger than the males, and sex ratios favor females at older ages, with a higher natural mortality rate for the males (Witherell & Burnett, 1993).

Winter Flounder Spawning

Winter flounder gonads ripen in the fall, and they begin moving into estuaries to prepare for spawning (Bigelow, 2002). In the spring of 2016, GOM winter flounder were sampled to better understand their spawning locations: inshore vs. offshore (Fairchild, 2017). Winter flounder were caught by trawls at three locations: southern Jeffreys Lodge, Bigear, and a portion of Stellwagen Bank National Marine Sanctuary. Sampled winter flounder included mature at total lengths greater than 28 cm. Because of the sites where ripe, running ripe, and recently spawned female flounder were caught, with distances of around 15 km from shore, it seemed improbable that they were migrating great distances to spawn (Fairchild, 2017). According to Fairchild, the energy required to travel those distances would not be energetically possible. It is often thought that winter flounder move inshore to spawn, but this study reveals that some flounder were spawning in deeper waters. It is important to better understand these spawning locations, and if they

are mostly living and spawning in fixed areas. It is unknown how winter flounder exposure to chemicals in those waters may have an effect on reproduction and/ or sex determination.

Winter flounder fall under these various stages of maturity: immature, developing, ripe, running ripe, spent, and resting (NOAA, n.d.c). The three that are especially important in better understanding winter flounder spawning locations are: ripe, running ripe and spent. Ripe is when nearly-mature eggs are present throughout the gonad, running ripe is when mature eggs have ovulated and are ready for release, and spent refers to the time immediately after spawning (NOAA, n.d.c). Because spawning is energy intensive, it is most likely occurring in the same general area where winter flounder in these three reproduction stages are caught (Fairchild, 2017).

Water Temperature Effects on Winter Flounder

Lethal temperatures for winter flounder are -1.4°C (or lower) and 19.3°C (or higher) (Bigelow, 2002). Flatfish species are influenced by temperature, pH and other environmental factors in combination with genetic factors that determine their sex, skewing the female to male ratio (Fairchild, Rennels, & Howell, 2007). In lab studies, winter flounder showed a male-skewed sex ratio that may be dependent on water temperature (Howell, Fairchild, & Rennels, 2014). Extreme water temperatures, low or high, yielded male-skewed sex ratios, while normal temperatures produced a 1:1 sex ratio (Luckenbach, Borski, Daniels, & Goodwin, 2009). Warmer water temperatures seem to increase rates of egg and larval development (Laurence, 1975), but survival rates are lowered (Rogers, 1976). Adult winter flounder seem more susceptible to changes in water

temperature, with juveniles showing more tolerance to high temperatures (Buckley, Moran, National Wetlands Research Center [NWRC], & U.S. Army Engineer Waterways Experiment Station [WES], 1989).

Cultured winter flounder populations showed a higher ratio of males, most likely due to the water in rearing tanks being several degrees warmer, or colder, than in nature (Howell et al., 2014). In fact, in another study of reared winter flounder in temperatures at 2°C, 5°C and 8°C (based on typical local estuarine temperatures in Narragansett Bay) found that both survival and growth were greater with increased temperature, with larvae not surviving to metamorphosis at 2°C when exposed to those temperatures for a long period of time (Laurence, 1975). However, it is unlikely that larvae would be exposed to such low temperatures for such a long period of time, or about 180 days until metamorphosis. It does seem to indicate that prolonged exposure to cold temperatures would negatively affect winter flounder growth (Laurence, 1975).

The winter flounder population sampled by the MWRA in Massachusetts Bay is predominantly female (Massachusetts Water Resources Authority [MWRA], 2016a). There may be a connection between water temperature and predator/ prey behaviors, resulting in lower landings of winter flounder in areas where overfishing is not occurring. This MWRA study suggests that temperature increases do not have a direct role in the historical decline of winter flounder, but that another driver, such as altered behavior in winter flounder feeding and reduced survival from predators, may indirectly be causing a decline after a critical threshold in temperature is reached (Able, Grothues, Morson, & Coleman, 2014). Water temperature affects oxygen consumption and metabolism in winter flounder, with higher temperatures resulting in higher consumption and metabolic

rates (Laurence, 1975). Although temperature from Boston Harbor to Stellwagen Bank varies seasonally, the warmer bottom water occurs in October (Geyer, 1999), which is generally a period just before spawning starts for winter flounder.

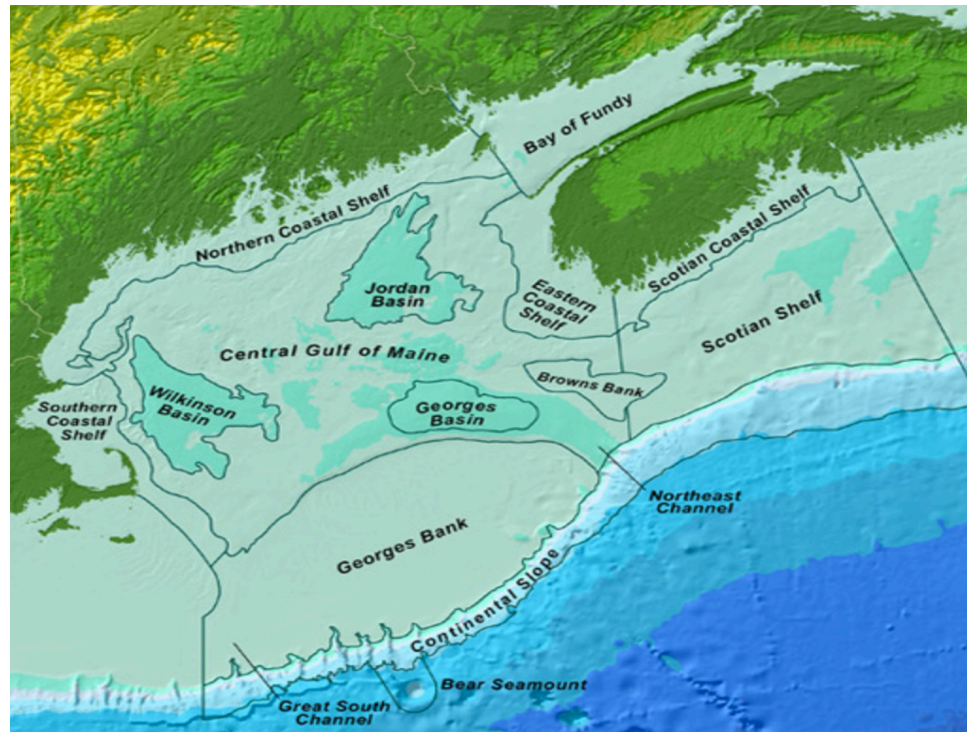


Figure 2. Gulf of Maine physioregions including the Southern Coastal Shelf (GoMA, 2018).

Southern Coastal Shelf

The Southern Coastal Shelf stretches over 3000 square miles, (GoMA, 2018) (Figure 2). It harbors the densest population of winter flounder in the GOM and is highly impacted by human activities (Gulf of Maine Area: Census of Marine Life [GoMA], 2018). The area is self-contained due to the way that the shallow parts of Stellwagen Bank cut it off from the rest of the Gulf of Maine. Currents that trap nutrients here also trap contaminants. How effluent plumes behaves in these conditions also depends upon the density of the ocean water, with temperature and salinity playing an important part in

either making seawater lighter or heavier (Massachusetts Division of Marine Fisheries [DMF], 2010). For this reason, it is important to look at winter flounder in the context of the entire Southern Coastal Shelf. Depths vary from the intertidal zone to about 100 meters, with sediments ranging from coarse-grained to finer silt and clay in deeper areas.

The Southern Coastal Shelf lies within the Gulf of Maine, and its circulation pattern, as well as the one in Massachusetts Bay, is generally counterclockwise (Geyer, 1999). This would mean that the circulation from the area of Boston Harbor's Deer Island treated sewage outfall moves down and eastward toward Cape Cod Bay. The seafloor in New England is greatly influenced by northeasters, or strong coastal storms, identified through surface-wave-generated bottom stress, causing the resuspension of bottom sediment (Butman, Sherwood, & Dalyander, 2008). The outfall is located in an area that is highly influenced by several factors that include sediment turbulence and transport. MWRA states that these are the main reasons that benthic habitat quality is high in the nearfield area, <5km from the outfall (MWRA, 2017b). But it is important to consider how circulation patterns and northeasters spread effluent effects to other areas.

Pharmaceuticals in the DIWWTP Effluent

The MWRA has found no evidence of pharmaceuticals in drinking water supplies but admits that compounds currently exist in wastewater (MWRA, 2015). The DIWWTP outfall is located 9.5 miles NE of Deer Island in 100-foot deep waters and was built to improve the water quality of Boston Harbor. Prior to 2000, the effluent was released at several locations just off of Deer Island and Nut Island in Boston Harbor (MWRA, 2015). The current outfall has been operating since September 2000, and effluent is released into

Massachusetts Bay from more than 50 diffuser heads, each with 8 small diffuser ports. According to the DMF, the outfall is not likely damaging surrounding habitats (DMF, 2010). Of the originally designed 55 diffuser heads (running in a straight line about 1¼ miles long), only 53 were operational at the outset of the new outfall location (K. Keay, MWRA personal communication). Although water quality has improved in the harbor, the new location in Massachusetts Bay may still pose various risks to marine life due to exposure to PPCPs, such as fluoxetine and estrogen.

The MWRA conducted a small PPCP (pharmaceutical and personal care products) study back in 2007, taking influent (wastewater flowing into the treatment center) and effluent (wastewater released from the treatment center) samples from DIWWTP in August and September of that year (M. F. Delaney, Lab Director MWRA, personal communication). This study was conducted to collect two sets of samples during dry-weather from influent, primary effluent (PREF), secondary effluent (SEEF), and final effluent (FEFF); these represented increasing levels of treatment before release. Their results revealed that wastewater treatment from influent to effluent removed above 80% of chemicals. Reported concentrations were in parts per trillion (ng/L, nanograms per Liter).

Thirty-one PPCPs were tested, including fluoxetine, acetaminophen, estrogen, flame retardants, and endocrine disrupting pesticides. Eleven chemicals were consistently detected but showed removals from influent to effluent at above 80%. None of these PPCPs are regulated by the EPA in recommended water quality standards. Fluoxetine levels were detected at 36 ng/L in August's FEFF, and esterone was 57 ng/L. Treatment barely lowered levels of carbamazepine in the August samples, from influent levels

detecting 290 ng/L, to FEEF of 240 ng/L. Other PPCPs detected in August and September FEEF included acetaminophen, ibuprofen, progesterone, iopromide, triclosan, tris (2-butoxyethyl) phosphate, phenol, and caffeine. This study revealed that all sorts of PPCPs end up in the ocean, in various concentrations, including flame retardants, antidepressants, antifungal and antibacterial agents, hormonal drugs, and pain relievers.

Since 2007, there has not been a similar study conducted by the MWRA that monitors pharmaceuticals in DIWWTP effluent. Although these results regarding pharmaceuticals in wastewater samples are readily available to the public, an investigation has not been conducted to better understand the repercussions of APIs being introduced into marine ecosystems. In addition to harmful effects from individual APIs, a combination and bioaccumulation of these pharmaceutical compounds may pose an even greater risk to marine life.

Effects of Pharmaceuticals on Fish Species

Several studies have established that fish uptake APIs, and that these chemicals have significant effects. After being exposed to small doses of Prozac, at one part per billion (at levels commonly found in wastewater effluent), male minnows in a lab were observed ignored females (Bienkowski, 2013). They were exposed early on in their development, around a few months old. Even when exposed to low concentrations, the males had decreased reproduction, took more time to capture prey, and hid from predators. (Bienkowski, 2013).

Rainbow trout were exposed to undiluted effluent from a Swedish municipal sewage treatment plant for a period of 13 days, and examined for antidepressant

bioconcentration (Grabicova et al., 2014). Citalopram, sertraline and venlafaxine were found in the brains and livers of most of these fish, and venlafaxine was found in the plasma. Antidepressants are commonly prescribed in the U.S., and appear regularly in contaminated waterways, possibly having an effect on fish in the wild. When male fathead minnows were exposed to higher levels of Prozac, but still at levels often detected in wastewater, females produced less eggs and males exhibited aggressive or deadly behavior towards the females (Bienkowski, 2013).

In Nebraska, samplers were installed in a river at five locations upstream and downstream of treated wastewater effluents (Bartelt-Hunt, Snow, Damon, Shockley, & Hoagland, 2009). They revealed that WWTP effluent was a significant source of pharmaceutical loading into those waters, including azithromycin, 1,7-dimethylxanthine, carbamazepine, cotinine, and sulfamethazine at all but one of the sampling locations. Water samples were collected from the effluent-impacted Niagara River in the Great Lakes, and found to contain the antidepressants citalopram, paroxetine, sertraline, venlafaxine, bupropion, and their metabolites norfluoxetine and nortriptyline (Arnnok et al., 2017). Concentrations in fish, due to exposure from effluent, were highest in the brain, as well as the liver, muscle and gonads. Antidepressants accumulate in bed sediments (Schultz et al., 2010), which makes this relevant for winter flounder since they mostly live in the benthic zone. Some fish, like the common rudd, tolerate polluted conditions better than other fish species. It is thought that some of these fishes are exposed to PPCPs through direct exposure through the gill area, rather than ingestion through diet (Arnnok et al., 2017).

In the wastewater effluent study of Shultz et al. (2010), white sucker populations in Boulder Creek, Colorado and Fourmile Creek, Iowa revealed that antidepressant uptake into brain tissue was occurring from water and/ or sediment exposure. It was unclear if this exposure was resulting in adverse effects to the health and reproduction of the fish. This study also discovered that fish uptake of antidepressants differs from those observed in water and sediment samples (Figure 3). Concentrations of venlafaxine were higher in sediment and water samples than they were in fish, while norfluoxetine, sertraline, and nortsertraline were much higher in the fish (Schultz et al., 2010). Because of the fact that antidepressants were designed to have an effect on brain chemicals in humans, the brains of fish are the most likely

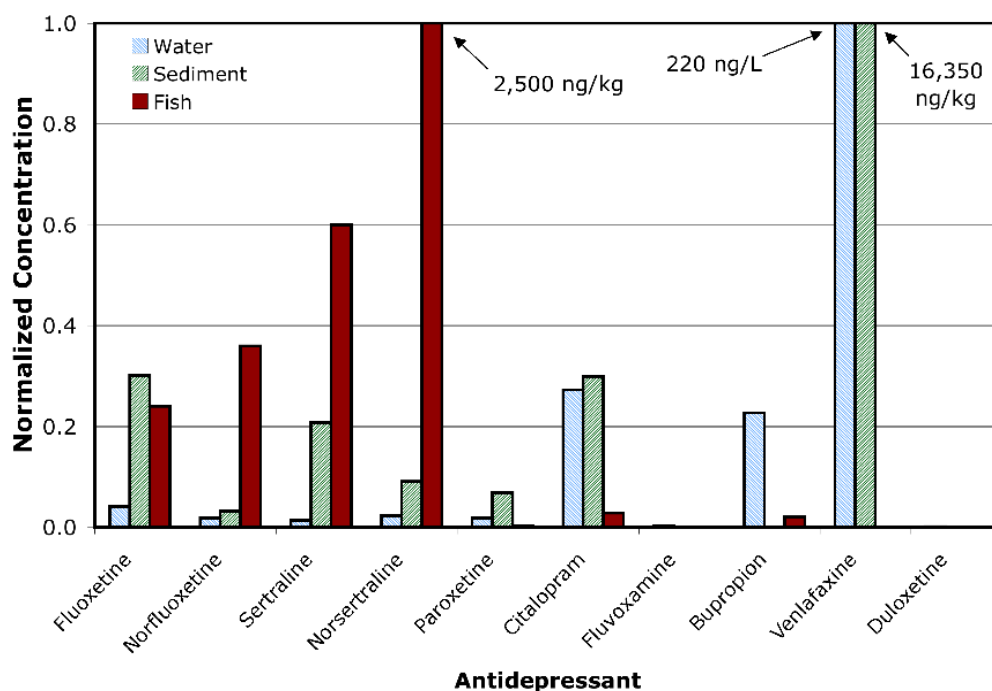


Figure 3. Concentrations of various APIs in water, sediment and fish from the Boulder Creek site in 2005 (USGS, n.d.).

place to be affected by exposure to these specific APIs (United States Geological Survey [USGS], n.d.).

Behavioral Changes

Antidepressants alter normal behaviors in marine species and could affect population levels. In the benthic marine amphipod, *Echinogammarus marinus*, antidepressants like fluoxetine caused the organism to spend more time in the light, higher in the water column, making them more vulnerable to predators (Guler & Ford, 2010; Bossus, Guler, Short, Morrison, & Ford, 2014). These behavioral changes occurred even when the amphipod was exposed to antidepressants at concentrations as low as 0.001 µg/L. Both geotaxis (motion in response to the force of gravity) and phototaxis (bodily movement in response to light, either away or toward it) were altered when exposed to serotonin re-uptake inhibitors (SSRIs). Serotonin controls many biological functions including reproduction, metabolism, and behavior (Bossus et al., 2014). Fluoxetine and sertraline are two of the most prescribed SSRIs, and *E. marinus* was exposed to both during this study, at environmentally relevant concentrations. There were observable behavioral changes, as this benthic organism that naturally favors dark regions was now seeking out the light.

The fathead minnow is often used in these types of lab studies and are present in all parts of North America. Juvenile and adult fathead minnows were exposed to municipal WWTP effluent, along with individual or mixtures of APIs, including oxycodone, fluoxetine, tramadol, and hydrocodone (USGS, 2015). Juvenile minnows grew slower and altered behaviors for escaping predators, by slowing down their reflexes

in dangerous situations and making them more susceptible to being caught. Adult females exposed to APIs had larger livers than the control minnows, suggesting that the liver was reacting to the exposure. The defense mechanism in males also seemed affected by the exposure, evident when they could not efficiently protect their nests. Males exposed to APIs also produced the chemical known as plasma vitellogenin (VTG), and in normal conditions VTG would only be present in females for egg production (USGS, 2015).

Egg Production

In a 17-stream study of fathead minnows, egg production during the 21 days of study was negatively correlated to urbanization and positively to wetland habitats (Crago, Corsi, Weber, Bannerman, & Klaper, 2011). This study discussed the importance of understanding how environmental factors, specifically water quality, affect fish populations. The research examined liver VTG mRNA as a predictor to egg production. Glutathione-S-transferase (GST) is a stress biomarker that is used in toxicity studies to provide information about an organism's stress responses to a pollutant, and low dissolved oxygen seemed to negatively affect egg production. Fish larvae may be exposed to contaminants while in the egg, from food or water consumed during development or after hatching, possibly causing mutagenic effects (Hylland, Feist, Thain, & Forlin, 2003). Sexual determination seems to occur prior to reaching 41 mm in total length (Fairchild et al., 2007). This would suggest that exposure to contaminants early in a fish's life could have an impact on sex determination.

Adult male and female fathead minnows were exposed to the following effluent concentrations: 0% (control), 5%, 20%, and 100% (Cavallin et al., 2016). Fecundity was

significantly reduced in the 100% effluent group, but increased in the 20% group. The first week of exposure resulted in the most noticeable effects on egg production, resulting in no spawning in 100% effluent, and higher production in 20% effluent. Week two revealed an increase in egg production in 100% effluent, and 20% effluent was similar to the control group. By the third week, all treatments had similar effects on fecundity. 100% effluent that had lowered fecundity, also resulted in a reduction in the number of daily spawns per female (Cavallin et al., 2016).

In a sampling program in the New York and New Jersey Harbor from 2002-2011, 3,033 epibenthic samples were collected examining the relationship between winter flounder egg and larval distributions with environmental factors (Wilber et al., 2013). Water temperature and sediment characteristics played a significant role with egg distributions, with colder temperatures resulting in delayed egg development. Warmer winter temperatures quickened egg development, but lowered survival rates and caused a reduction in yolk-sac larval size (Laurence, 1975; Rogers, 1976). Winter flounder egg densities were found to be highest in shallow habitats (Wilber et al., 2013).

Endocrine Disruption

Endocrine disruption is known as the interference in the normal functioning of the hormone system from environmental chemicals. UK estuaries contaminated with estrogens caused biological effects in flounder, including VTG production in males, and altered normal breeding patterns (Allen et al., 1999). Inappropriate feminization of UK freshwater and marine male fishes occurred when exposed to estrogens from sewage effluent or urbanized coastal waters (Matthiessen et al., 1998). The European flounder,

Platichthys flesus, was studied due to its spawning habits. These flatfish tend to spend most of their life in rivers or estuaries, and only head offshore for breeding. Since it is bottom-dwelling, the flounder is exposed to various contaminants in the sediment. The feminization would mean that egg cells develop in normal testes, and this process may affect how these fish breed. Studying the locations of the discharges in relation to the feminization in flounder will help explain possible effects. Large numbers of synthetic chemicals are found in industrialized estuaries, having the most significant effects in flounder, and ovotestis is most likely induced through exposure of flounder larvae during the stage of gonadal development (Matthiessen et al., 1998).

Male juvenile winter flounder from coastal urbanized areas in NY have shown signs of feminization, but this has not been similarly analyzed in wild caught juvenile flounder (MWRA, 2016b). Male fish exposure to non-hormonal APIs, such as metformin, have shown to cause an induction of testis-ova (intersex) in gonads (Niemuth & Klaper, 2015). Male rainbow trout exposed to estrogenic chemicals have also exhibited changes in normal sexual development (Jobling, 1995). Exposure to these chemicals were thought to have subtle but serious effects on their development. It is possible that reproduction is affected by exposure to contaminants during the production of offspring including but not limited to sex determination and gamete and embryo development (Gibson, 2005). Exposure to the contaminants could come through several means including diet and migration into more contaminated areas.

Male smallmouth bass in parts of the Potomac River and the Shenandoah River were observed having testicular oocytes (intersex), suggesting an exposure to endocrine disrupting chemicals (Iwanowicz et al., 2009). Intersex rates of 82-100% were recorded

at all sites, and proximity to effluent from WWTPs may have an influence on reproduction, but that other sources may also be contributing to the high incidences of testicular oocytes (Iwanowicz et al., 2009). Measuring VTG in male and female flounder helps search for feminized testes and other changes in sex (Matthiessen et al., 1998). But endocrine disruptors are not the only cause for concern. Pollution impacts reproduction in many ways, including in fecundity and the fertilization process, as well as altering natural behaviors in mate recognition and/ or attraction (Goksoyr et al., 2003).

Low concentrations of PPCPs from effluent, even into waters that have high dilution factors, could cause long term effects in aquatic organisms. These organisms are often exposed to various types of endocrine disrupting chemicals, in low doses, over time, and often a cocktail of various compounds (Sumpter & Johnson, 2005). It is necessary to discover how continuous exposure, at low levels, and in combination with other PPCPs, could alter marine organisms' natural behaviors and reproduction. Most concerning are the pharmaceutical compounds, that are biologically active often at very low concentrations (Sumpter & Johnson, 2005). Recruitment could be affected when fecundity and spawning ability are reduced, resulting in a decrease in the number of eggs (Hylland et al., 2003).

MWRA Monitoring of the Outfall and Marine Species

The MWRA has been monitoring winter flounder in Massachusetts Bay since 1991, after contaminant-associated liver diseases were detected in high numbers in Boston Harbor in late 1980's (MWRA, 2015). There were concerns about the ecological health of the Harbor when centrotubular hydropic vacuolation (CHV), a condition

associated with exposure to contaminants and a precursor to tumor growth, and tumors were detected in these flounder, and about possible repercussions for flounder exposed to effluent at the new outfall site. Monitoring was put into place to study these impacts and record any improvements. DIWWTP's permit to discharge into Mass Bay was contingent on the fact that the effluent would constantly be monitored.

Annually, the MWRA monitors the health of winter flounder from four locations: the outfall site (OS) in Mass Bay, Deer Island Flats (DIF) in Boston Harbor, off Nantasket Beach (NB), and East Cape Cod Bay (ECCB) (MWRA, 2016a). The samples collected at these locations are included in reports documenting chemicals detected in flounder fillets and liver, lobster meat and hepatopancreas, and blue mussels. In order to detect liver effects, MWRA has determined that only 30 cm and larger flounder would be studied. These results have been available to the public since 1992, and include monitoring on the benthos/ seafloor, water/bays, and effluent (MWRA, n.d.).

The 2017 MWRA flounder monitoring is the 17th consecutive year since the start of the new outfall location in September 2000, and concentrations of organic and inorganic contaminants in body tissue are assembled every third year (MWRA, 2017c). In addition to collecting information about the presence of disease, winter flounder were sampled for biological data that include size, age and gender. All four locations sampled had a high percentage of female winter flounder (MWRA, 2016a). The technical reports also document worrisome events like red tide and also the sex ratio of winter flounder in the samples (MWRA, 2015).

Benthic Monitoring

Winter flounder are susceptible to disturbances in the seafloor as a result of trawling or dredging, altering their habitat and affecting the survival of juveniles (Gibson, 2005). Understanding the benthic ecosystem is critical in order to determine causes for lower seafood landings, despite the fact that overfishing is not occurring. The population cannot rebound if changes in water quality are preventing winter flounder from reaching its potential (Maine.gov, 2016).

MWRA conducts benthic monitoring of sediments and soft-bottom communities. These surveys are conducted using traditional grab sampling and sediment profile imaging (SPI), and a remotely operated vehicle (ROV) surveys hard-bottom communities (MWRA, 2017b). When the outfall was moved to its current location in Massachusetts Bay, there were concerns about effects from effluent on benthic communities. Although benthic sampling does not include an examination of pharmaceutical compounds, it does focus on three areas: eutrophication and low levels of dissolved oxygen (DO), toxic contaminants, and particulate matter affecting animal survival. Based on their results, they concluded that none of these factors were affected at the sampled stations. Annual monitoring includes soft-bottom sampling for sediment and infauna conditions at 14 nearfield and farfield locations, and SPI at 23 nearfield stations. Every third year these same stations are evaluated for sediment contaminants (MWRA, 2017b).

Effluent impact is better understood when looking at concentrations of *Clostridium perfringens*. *C. perfringens* is an anaerobic bacterium that can be traced back to effluent solids (MWRA, 2017b). Post diversion, there were increases in concentrations of this bacterium within 2 km of the effluent (Figure 4). MWRA reports state that since

the startup of the outfall, the three impacts of primary concern (DO, toxic contaminants, and particulate matter on animal survival) had not occurred at their stations. However, increases in concentrations of *C. perfringens*, as well as higher diversity and lower dominance in benthic animals in Massachusetts Bay, are likely due to effluent discharge (MWRA, 2017b).

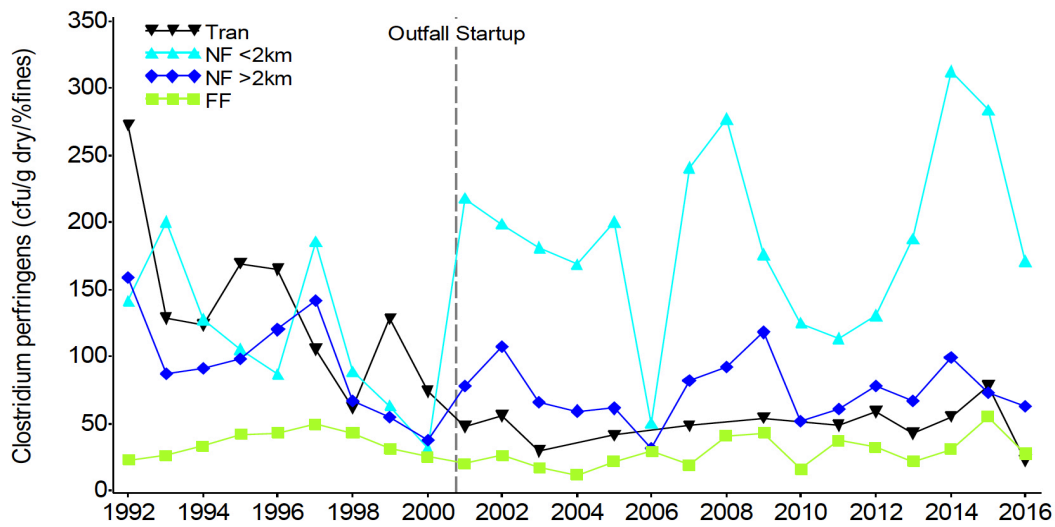


Figure 4. *Clostridium perfringens* mean concentrations in four areas of Massachusetts Bay. Tran= transition area (between Boston Harbor and outfall); NF<2km= nearfield, less than 2 km from outfall; NF>2km= nearfield, more than 2 km from outfall (but <5km); FF= farfield, greater than 13km from outfall (MWRA, 2017b).

During the baseline period, NF<2km had *C. perfringens* concentrations between 50-200 cfu/g dry/% fines. The outfall location in Massachusetts Bay became active in September 2000, and the levels of *C. perfringens* (Figure 4) clearly show effects from the effluent. Levels in the transition area, in the vicinity of the Boston Harbor outfall site, had the steepest decline since 2001. NF>2km did not show any observable trends from 1992-2016. FF levels hardly changed during this same period. The greatest changes occurred in the Transition and NF<2km areas. During the baseline period, Boston Harbor

experienced a decrease in eelgrass areas, and an increase since the diversion of the outfall to Massachusetts Bay due to improvements in wastewater treatment in general (MWRA, 2017a).

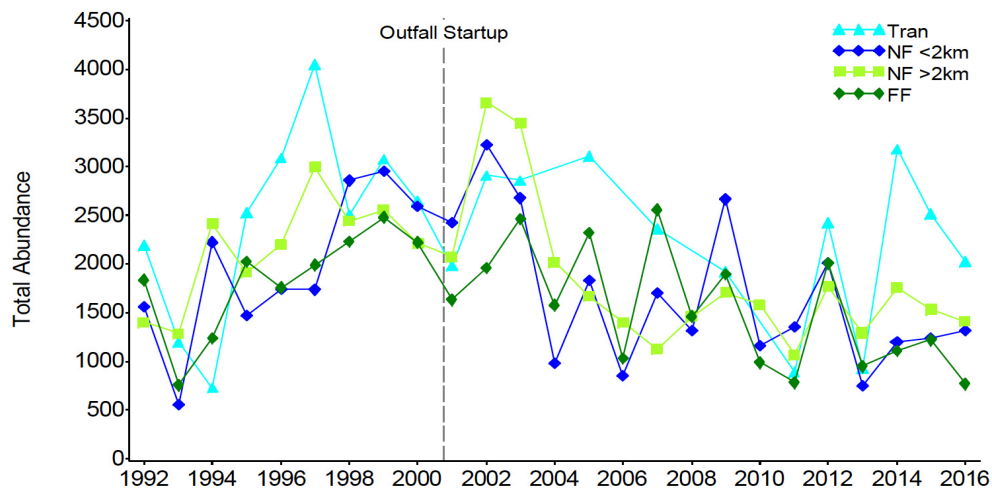


Figure 5. Massachusetts Bay mean infaunal abundance per sample (MWRA, 2017b).

Infaunal abundance in Boston Harbor and Massachusetts Bay may play an important role in understanding winter flounder population decreases and changes to commercial landings (Figures 5 & 6). Infaunal abundance per sample seems to show a slight increase immediately following the Mass Bay outfall startup in 2000, followed by a sharp decline in abundance in the NF<2km area (Figure 5). Most of the other areas exhibit similar trends. Small benthic species are critical in the diet of winter flounder. Declines in their abundance in Boston Harbor and in Massachusetts Bay (Figure 6) is most likely one of the contributing factors to lowered winter flounder commercial landings.

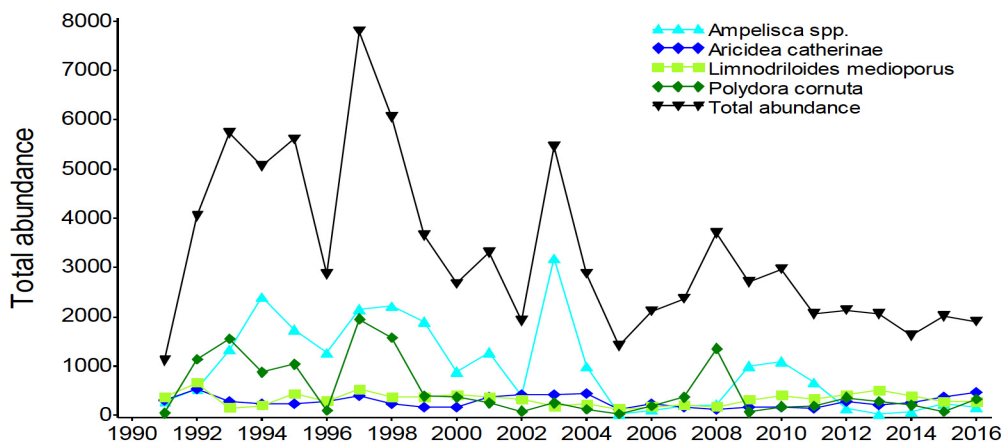


Figure 6. Boston Harbor total abundance of four dominant taxa (MWRA, 2017a).

Winter Flounder Sampling and Sex Ratio Results

Until 2007, the MWRA sampling was conducted by commercial otter trawls towed by commercial dragger F/V Odessa vessel (70' sweep rope) (MWRA, 2017d). Since 2008, the F/V Harvest Moon (74' sweep rope) has taken over (except for one station in 2008 that used the F/V Explorer 2 with an 84' sweep rope). Surveys are carried out in the spring, with tows at each station lasting anywhere from 30-60 minutes at speeds of 1.5 to 2 knots. Standard operating protocol for the dissection of winter flounder include opening the blind side of the visceral cavity to examine the liver, and cutting sections that include lesions. Gender and sexual maturity are confirmed by examining gonads (MWRA, 2016a). Dr. Michael Moore (Woods Hole Oceanographic Institution [WHOI]) is the principal investigator for flounder monitoring and prepares the annual reports with the flounder tissue analysis results. Additional information is found in the Quality Assurance Plan (MWRA, 2017d).

It is important to note that MWRA's monitoring only looks at winter flounder ≥ 30 cm in total length. They currently do not monitor flounder under 30 cm, and their

monitoring is limited to a few locations. MWRA monitors the health of Boston Harbor and Massachusetts Bay, looking at water quality, fish and shellfish chemistry, and sediment contaminants. Monitoring for each report is conducted at the same general locations. Sampling of winter flounder takes place at four specific sites (Figure 7), with 50 sexually mature winter flounder collected at each location. These four locations are: Deer Island Flats (DIF), off Nantasket Beach (NB), East Cape Cod Bay (ECCB), and Outfall Site (OS). These reports potentially reveal the effects of contaminants in the environment on winter flounder ≥ 30 cm, since larger and older fish better reveal long-term effects of exposure in the liver (MWRA, 2017c).

MWRA's OS, or outfall site, is not located at the actual outfall (Figure 7). OS is the closest consistently trawlable bottom area to the outfall. The location used for the OS is between .91-2.14 miles from various points of the outfall diffusers. OS was determined a suitable sampling location by Dr. Michael Moore of Woods Hole Oceanographic Institute, PI of the flounder study, and coastal fisherman with whom he helped create the sampling design in the early 1990's (K. Keay, MWRA personal communication). The distance consideration, as well as the fact that female winter flounder grow longer than the males (and MWRA only include winter flounder ≥ 30 cm), are important when trying to understand their sex ratio results. Their results would naturally reveal a skewed sex ratio favoring females, especially when almost half the flounder are 36 cm and above. Therefore, it is important to check if smaller mature fish in the group (30-35 cm) similarly yield a high percentage of females.

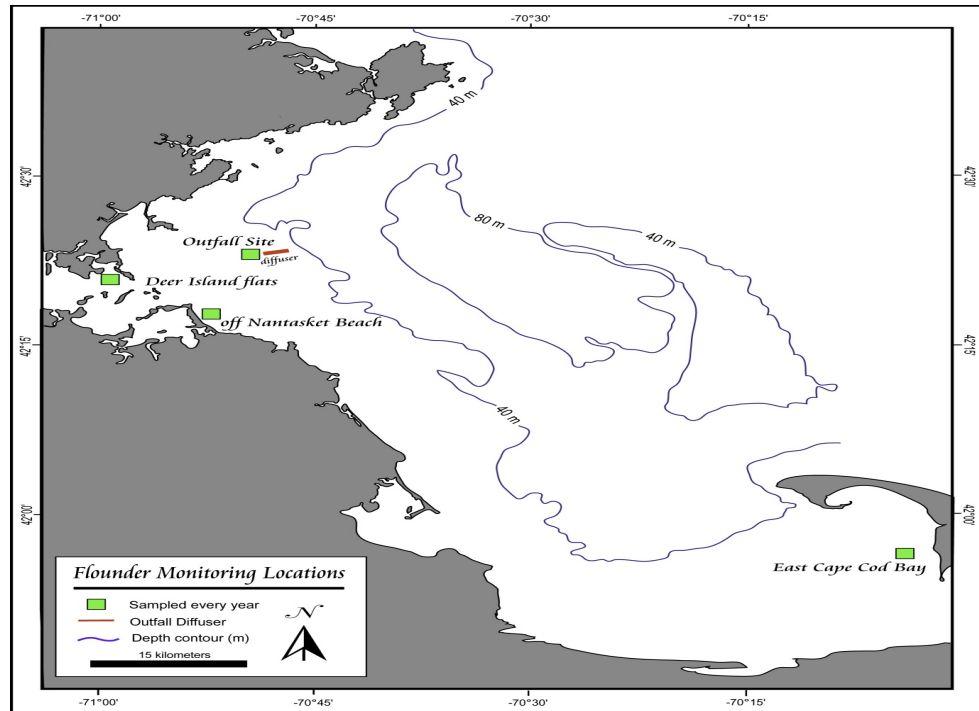


Figure 7. MWRA locations for long-term monitoring of flounder (MWRA, 2017c).

MWRA's sampled populations of winter flounder do not reveal major post-diversion changes in the percentage of females at the outfall site (Figure 8). Based on the monitoring results, there seemed to be an increasing trend in % females in the sampled population of winter flounder from 1991-2001, but no observable trend based on distance from the outfall. In fact, sampling locations, such as OS, have percentages that have stayed about the same when comparing the baseline period to the post-diversion period. As mentioned previously, this monitoring only includes winter flounder ≥ 30 cm. Based on known growth and maturity patterns for winter flounder (females grow larger than males), it is expected that the ≥ 30 cm data result in an overall high percentage of females.

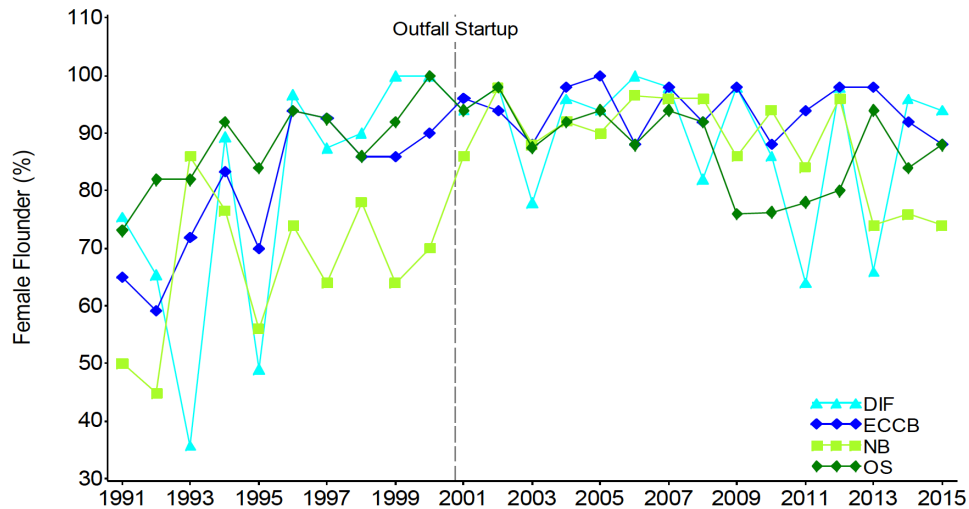


Figure 8. MWRA proportion of female winter flounder compared by station and year (MWRA, 2016b).

Disease Prevalence

Centrotubular hydropic vacuolation (CHV) was highest off Deer Island Flats (DIF), and lowest at the outfall site (OS) (MWRA, 2016b). The prevalence of neoplasm from DIF fish in the mid to late 1980's has disappeared, and has never been observed in any of the fish from the outfall site. The recent samples of winter flounder range from 66% to 98% female, and those percentages are higher post-diversion (Figure 8). MWRA has not identified an association between changes to the outfall location and the increase in rates of female winter flounder (MWRA, 2016b).

Lymphocystis is a well-known viral infection in marine and freshwater fishes that could interfere with a flounder's eating habits, making it lethargic (Eiras & Eiras, 2008). In addition, lymphocystis could interfere with the fish's ability to swim if its fins and gills are covered with large nodules. Water pollution and stress factors lead to an increase in infection. If there are many lesions and nodules on the gills and other important organs, the fish is more likely to contract a secondary infection by bacteria or parasites, likely

leading to mortality (Yanong, 2016). Lesions on gonads may also affect reproduction, and recruitment could be reduced even if a portion of the population is affected (Hylland et al., 2003).

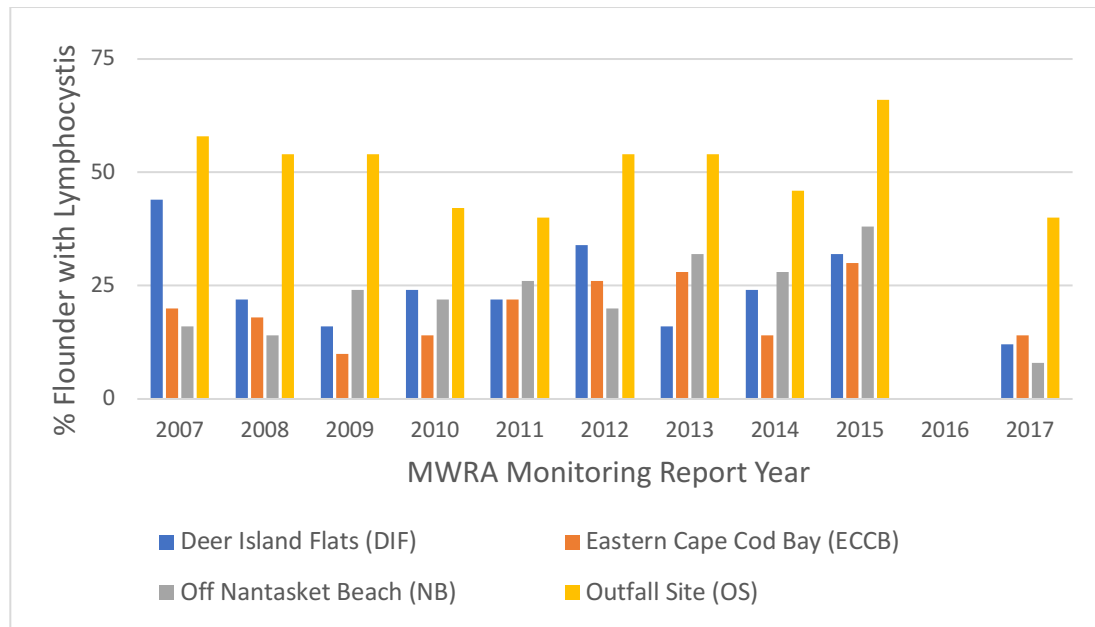


Figure 9. Winter flounder lymphocystis prevalence by MWRA station. Sample Size n=50 per location. Data not available for 2016. Data compiled from 2007-2017 MWRA winter flounder monitoring reports.

In the 2015 Fish and Shellfish Tissue Chemistry Report, lymphocystis rates were as high as 66% at the OS in a sample size of 50 (MWRA, 2016a). In fact, flounder closer to the OS are exhibiting a greater prevalence for lymphocystis compared to any other station (Figure 9). In 2017, the prevalence of lymphocystis at OS decreased to 40%, but the lowest observed level was 8% at NB (MWRA, 2017c). Lymphocystis data are not available prior to 2007, but it was never detected in the 1980's. In addition to the MWRA stations listed above, high incidences of lymphocystis were also observed near Burnam Rocks (GPS 42°31.32', -70°40.60') and east of Eastern Point (GPS 42°35.48', -

70°34.88') (Moore, 2003). Both of these points are located north of the MWRA Mass Bay outfall.

In 2003, the prevalence of blind side ulcers was recorded in 24% of winter flounder sampled at the outfall site, but it is not possible to determine the cause for the ulcers (Moore, 2003). During that same time, Deer Island samples had between 20-27% external ulcers, with no similar detections made at Eastern Cape Cod Bay. There were no clear reports of ulcers prior to 2003, but levels have fluctuated since then, and by 2017, ulcers were absent on all fish (MWRA, 2017c).

Federal and State Monitoring and Sampling Protocols

The DMF (state agency) conducts yearly sampling of winter flounder. Stations are randomly assigned within each stratum, with one station per 19 square miles, and 100 stations per survey (DMF, 2015). Sampling is conducted in September (as juveniles prepare to migrate) and in May (when adults arrive to spawn). Since 1982, the DMF has used the vessel R/V Gloria Michelle, with a ¾ size Yankee trawl. Surveys include 20-minute tows at 2.5 knots. Inshore bottom trawl surveys cover five geographic regions, with up to six depth zones per region (DMF, 2015).

Similarly, the NEFSC (federal agency) conducts yearly monitoring of winter flounder. They used Albatross IV until 2008, followed by Henry B. Bigelow as their primary research vessel. The current vessel utilizes as their primary sampling gear a standardized 3-bridlle, 4-seam bottom trawl survey with a rockhopper sweep (NOAA, 2014). NEFSC has been conducting bottom trawl surveys along the U.S. northeastern continental shelf since 1963. Employing a sampling design within each stratum, trawling

locations are randomly selected prior to each cruise. Bottom trawling at each location lasts 20 minutes and towed at 3.0 knots as soon as the trawl first touches the bottom, towing in the direction of the next sampling station.

For both the NEFSC and DMF sampling, two flounder are chosen at random per size group per station. Their sampling protocols are almost identical. They record their data into the Fisheries Scientific Computer System (FSCS), and sex determination is made visually (NOAA, 2016). The MWRA sampling research will be used for comparison only to the raw data collected by the NEFSC and the DMF, looking at flounder sizes 30-35 cm.

Winter Flounder Commercial Landings

U.S. commercial trawl fisheries (for flatfish such as the winter flounder) were developed from small fishing vessels in the first half of the 20th century and then to much larger diesel- and gasoline-powered vessels (Gibson, 2005). Commercial landings of winter flounder peaked in 1987 with over 24,000,000 pounds, and dropped to as low as 3,700,000 by 2015. In 1996 a new law came into effect, the Sustainable Fisheries Act, which prevented overfishing and established quotas (Fissues, 2017). NEFSC collects data on the total seafood landings on various species, including winter flounder, and the annual landings show a steady decline in Massachusetts from 1980 to 2017 (Figure 10) (NOAA, n.d.a).

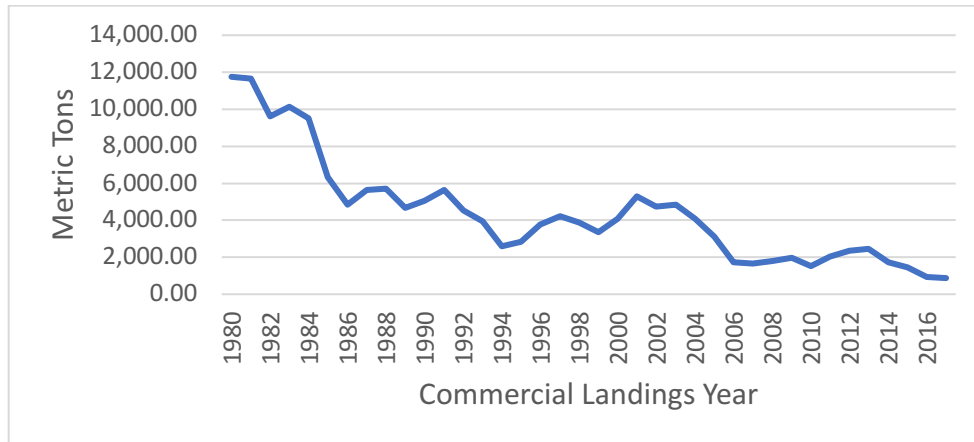


Figure 10. NMFS winter flounder annual landings, Massachusetts. Winter flounder annual landings in Massachusetts compiled from NMFS data available online (NOAA, n.d.a).

Winter flounder recovery is slow due to fishing pressure, habitat degradation and low genetic variability, with various factors such as temperature, salinity, dissolved oxygen and food availability affecting larval and juvenile development (ASMFC, n.d.). Landings have dropped from 11,752 metric tons in 1980, to just 873 metric tons in 2017.

The management of winter flounder in federal waters is the responsibility of NOAA fisheries and the New England Fishery Management Council, while the Atlantic States Marines Fisheries Commission manages those in states waters 0 to 3 miles offshore (NOAA, n.d.b). In 2010, Gulf of Maine and Georges Bank stocks were not experiencing overfishing. The Southern New England/ Mid-Atlantic (SNE/MA) winter flounder was overfished but not experiencing overfishing (ASMFC, 2014). In 2010 the spawning stock biomass for Southern New England/ Mid Atlantic was estimated to be about 7,076 metric tons, with GOM estimated at 1,516 metric tons. Massachusetts, Rhode Island, New York and Delaware are required under Amendment 1 of the Interstate Fishery Management plan to continue annual surveys of juvenile recruitment for winter flounder to develop an annual juvenile abundance index (ASMFC, 2014).

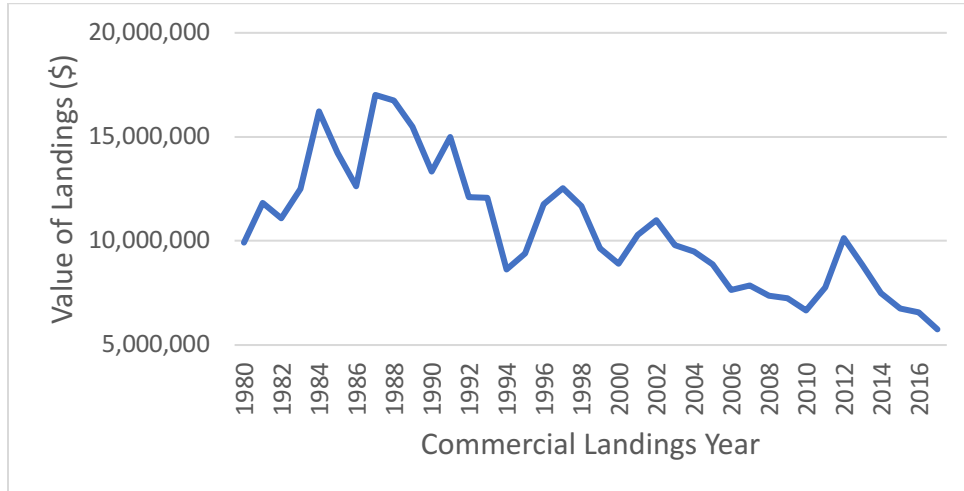


Figure 11. Commercial landings value (U.S. dollars) for winter flounder in Massachusetts. Commercial landings values compiled from available online data (NOAA, n.d.a).

Changes in commercial fisheries landings have a dollar value (Figure 11). The numbers come from seafood dealers who submit monthly reports of the weight and value of landings by vessel, or from trip-tickets filled out by seafood dealers and fishermen at the end of fishing trips that indicate landings (NOAA, n.d.a). The economic cost for these drops in economic value is significant in Massachusetts, from highs of over \$16 million in the 1980s, to a low of under \$6 million in 2017. There are clearly monetary consequences for failing to understand the causes of the decrease in commercial landings.

Research Question, Hypothesis and Specific Aims

My research was intended to evaluate this question: Is the declining sex ratio of winter flounder in Massachusetts Bay consistent with their exposure to pharmaceutical compounds from the Deer Island Treatment Plant effluent? Although overfishing or warmer water temperatures are possible explanations for lower commercial seafood

landings, APIs from the MWRA outfall may be contributing to the decline in the male population of winter flounder.

To help address this question, I examined the following hypothesis: winter flounder in Massachusetts Bay show a decline in sex ratio in relation to proximity to the DIWWTP outfall location.

Specific Aims

To test out this hypothesis, I:

- Mapped out GOM winter flounder sampling locations and their proximity to the current outfall site.
- Analyzed historical information and trends from MWRA, NEFSC and DMF on sex ratio composition for winter flounder.
- Explored the correlation between sex ratio and distance from effluent, looking at thousands of locations sampled from 1980-2017 by NEFSC & DMF within Boston Harbor and Massachusetts Bay. This was compared to winter flounder data collected annually by MWRA at their four sampling locations. GPS coordinates of sampling sites, as well as the MWRA outfall location, are entered into QGIS to determine possible trends in the ratio of female to male flounder.
- Examined spawning locations of winter flounder, using QGIS to plot locations of fish in the following reproduction stages: ripe, ripe and running, and spent.

Chapter II

Methods

Winter flounder MWRA sex data were collected during sampling at only four locations (DIF, NB, OS, ECCB). These data were used as comparison to the thousands of available raw sampling points from NEFSC (federal) and DMF (state) datasets.

Monitoring conducted by these agencies has not been an in-depth population-wide study of the gender composition of GOM winter flounder in Massachusetts waters, because of the various sampling protocols used by each of the agencies. MWRA randomly samples all winter flounder but limits the size of fish to at least 30 cm (total length). NEFSC and DMF, on the other hand, sample for each cm size class making sure that they collect at least one fish from each size category. Although NEFSC and DMF don't sample randomly by the entire tow, they do randomly select a fish per cm class.

The data were collated from both published and unpublished literature and data that are freely available to the public. QGIS (Quantum Geographic Information System) was utilized for a visual representation, to include GPS coordinates and sex data on individual flounder. The maps showed if there were any noticeable trends, before and after the startup of the Mass Bay outfall, and differences in sex composition by location. GOM winter flounder raw data from the National Marine Fisheries Service/ Northeast Fisheries Science Center (NMFS NEFSC) NOAA, and the Massachusetts Division of Marine Fisheries (DMF) were organized for analysis. NEFSC and DMF used almost identical sampling protocols, and therefore their data were combined. Altogether, 33,521

individual fish data were collected by these two agencies from 1980-2017. These were sorted and filtered by the following methods:

- Raw data from fall and spring surveys for NEFSC 1980-2000 & DMF 1994-2000 were combined into one Excel worksheet. This “baseline” period included all samples during the time when the outfall was located in Boston Harbor.
- Raw data from fall and spring surveys for NEFSC & DMF 2001-2017 were also combined. This “post-diversion” period included all samples since the outfall was moved in late 2000 to its present location in Massachusetts Bay.
- The baseline and post-diversion worksheets were each sorted to remove all non-winter flounder species, as well as winter flounder that could not be sexed.
- Because GOM flounder were sampled as far north as Maine, and to control for effects of the outfall, only flounder in Massachusetts Bay and Cape Cod Bay were kept. The individual GPS locations for flounder were filtered to only include those caught between longitudes 42°40’12” N and 41°45’0” N, and no further East than 70° W.
- Only winter flounder in seven size groups (total length in centimeters) were kept for distance calculations. These sizes are 29-35 cm, inclusive. This prevented smaller, immature fish, as well as larger ones that tend to be female, from skewing results. The decision to use 29-35 cm winter flounder also allowed for a more manageable number of mature fish to work with.
- Distance (in miles) was calculated using the haversine formula in Excel, between two sets of GPS coordinates: the sampled location of the individual winter flounder (beginning longitude and latitude of tow), and the midpoint of the outfall in Massachusetts Bay. The

midpoint of the outfall was used for the distance calculation because the outfall is over a mile long.

- Statistical tests were performed on the sex ratio data using <http://vassarstats.net/> to examine differences between two independent proportions.
- All sizes were considered only when examining maturity and possible spawning locations in Boston Harbor and Massachusetts. Size groups are based on the total length (TL) of the flounder, using the length recorded in the data from NEFSC and DMF sampling. After sorting and filtering was completed, 7,342 flounder remained (baseline n = 2,412, post-diversion n = 4,930). The baseline period had less winter flounder than the post-diversion period due to the fact that DMF's sampling started in 1994.

During each tow conducted by NEFSC and DMF, biological details on individual winter flounder included, but were not limited to, the following: date of tow, average depth, surface and bottom temperatures, season, beginning and ending latitude and longitude of tow, length, maturity and sex of the winter flounder. After winter flounder were sorted by distance from the outfall, they were arranged into their corresponding size group (29-35 cm). Distances were divided into the following 5 groups: <10 miles, 10<20 miles, 20<30 miles, 30<40 miles, and >40 miles. Although a group closer to the outfall would have been ideal (<1 mile), there were not enough fish sampled per cm in that range, even after all data from both agencies were combined. After the distance calculations were performed within the coordinates mentioned above, most of the remaining flounder 29-35 cm were well within 50 miles from the outfall location. Sex ratio was calculated for populations of winter flounder in each distance group.

NEFSC and DMF use similar sampling protocols, randomly sampling each cm, and not proportional to all fish in the tow. This is why size has to be factored into sex ratio analysis whenever using their data. MWRA data were only used as a comparison, because of differing sampling protocols. MWRA performs yearly sampling at only four locations, and mentions their closest and furthest location results on percentage female in relation to distance from the outfall. Their sampling was not conducted at enough stations within Massachusetts Bay to explain declines in the sex ratio. In addition, the “outfall site”, one of the four sampling locations, is not the actual outfall site but located 0.91 miles from the closest diffuser head (based on GPS distance calculations).

The various coordinates for the outfall’s first and last diffusers were used in QGIS, and the midpoint was used in Excel for the distance calculations (Table 1). Because the midpoint was used for the calculations, and since the outfall runs along a line stretching 1¼ miles, all distance results should account for a difference of ± 0.6 miles. The coordinates for the four MWRA sampling locations (Table 1) were planned locations. The actual location (coordinates) were very close to the planned location.

During the initial organization of raw data, it was immediately clear that none of the agencies were sampling winter flounder to understand the dynamics of sex ratio in the population. The research was made more difficult by the fact that each of the agencies used different sampling protocols, and certain locations yield low sample sizes, with especially low numbers near the outfall. Because NEFSC & DMF sampled flounder per cm, their raw data has to be organized and sorted by size prior to any analyzing of sex ratios for the GOM stock. MWRA data only include fish larger than 30 cm, and their results naturally skew in favor of females because larger winter flounder tend to be

female. In order to compare it to the NEFSC & DMF groups, MWRA samples needed to similarly be adjusted by size to leave out larger fish from the sex ratio calculations. This would allow for a better understanding of this particular stock. Since female flounder grow larger than males, the higher size categories were removed from MWRA's data in order to compare it to NEFSC and DMF data.

Table 1. MWRA sampling locations and DIWWTP Mass Bay outfall GPS coordinates.

Location	Latitude (N)	Longitude (W)
Outfall Site (OS)	42.385	-70.82167
Off Nantasket Beach (NB)	42.2933	-70.87
Deer Island Flats (DIF)	42.34	-70.9733
Eastern Cape Cod Bay (ECCB)	41.9367	-70.11
Outfall Diffuser #1 (easternmost)	42.388683	-70.78009
Outfall Diffuser #55 (westernmost)	42.384312	-70.803795
Outfall Midpoint (used for distance analysis)	42.386715	-70.791298

NEFSC and DMF GPS coordinates for individual winter flounder, distance calculations, maturity and sex data were loaded into QGIS and plotted. In addition, the outfall (55 diffuser heads) and MWRA's four sampling stations were added. An ESRI Ocean raster layer was used in QGIS as the background map. MWRA data were used as a comparison, since their sampling consisted of only four unique locations. In contrast, DMF and NEFSC include thousands of locations on individual winter flounder within state and federal waters off the coast of Massachusetts, and those numbers have not been similarly tested for distance from outfall and sex relationship.

Chapter III

Results

Immature fish in the smaller size categories had a more equal number of males and females in the population, and larger winter flounder were majority female, with almost 100% female in the highest sizes for both the baseline 1980-2000 combined samples (Table 2) and post-diversion 2001-2017 samples (Table 3). However, there is an overall increase in percentage females, even in the smaller size categories.

Both the 29 cm and 30 cm flounders showed a significant change in sex composition, pre- and post-diversion, with an 8% increase in females for each group (Tables 2 & 3). The proportion of females in the post-diversion 29 cm class was significantly greater than in the baseline sample ($z = -2.506$, $p = 0.0122$), similar to the result for the 30 cm class ($z = -2.694$, $p = 0.0071$).

The average for the 29-35 cm baseline winter flounder yielded 68% female for the population. The average for these same sizes post-diversion yielded 72% female. This gives an overall picture of the flounder per size category during the baseline and post-diversion periods.

Table 2. NEFSC & DMF 1980-2000 combined data on Gulf of Maine winter flounder, Massachusetts.

Winter Flounder Length (cm)	Winter Flounder Sample Size (n)	Female (n)	% Female
6	1	0	0%
7	4	3	75%
8	18	11	61%
9	31	18	58%
10	61	32	52%
11	56	37	66%
12	65	37	57%
13	88	38	43%
14	89	44	49%
15	89	46	52%
16	117	64	55%
17	139	53	38%
18	144	71	49%
19	170	74	44%
20	195	78	40%
21	205	105	51%
22	217	93	43%
23	228	110	48%
24	253	125	49%
25	279	151	54%
26	303	170	56%
27	314	169	54%
28	314	175	56%
29	334	187	56%
30	372	213	57%
31	359	240	67%
32	350	229	65%

33	338	252	75%
34	356	269	76%
35	302	250	83%
36	255	206	81%
37	191	165	86%
38	145	113	78%
39	124	115	93%
40	74	71	96%
41	46	43	93%
42	38	33	87%
43	24	24	100%
44	17	16	94%
45	20	19	95%
46	10	9	90%
47	8	8	100%
48	8	6	75%
49	9	8	89%
50	5	5	100%
51	4	3	75%
52	5	5	100%
53	3	3	100%
54	2	1	50%
55	3	3	100%
56	0	0	n/a
57	2	2	100%
58	1	1	100%
59	1	1	100%
60	0	0	n/a
61	1	1	100%
Total	6787	4205	71%

Table 3. NEFSC & DMF 2001-2017 combined data on Gulf of Maine winter flounder, Massachusetts.

Winter Flounder Length (cm)	Winter Flounder Sample Size (n)	Female (n)	% Female
6	3	1	33%
7	16	12	75%
8	42	24	57%
9	65	42	65%
10	128	79	62%
11	165	106	64%
12	207	113	55%
13	262	144	55%
14	324	186	57%
15	321	165	51%
16	446	251	56%
17	420	217	52%
18	497	257	52%
19	492	263	53%
20	547	280	51%
21	527	259	49%
22	600	315	53%
23	603	317	53%
24	666	324	49%
25	661	361	55%
26	669	358	54%
27	697	401	58%
28	730	430	59%
29	697	445	64%
30	765	501	65%

31	694	473	68%
32	688	489	71%
33	681	512	75%
34	689	552	80%
35	729	609	84%
36	627	545	87%
37	542	488	90%
38	418	392	94%
39	305	284	93%
40	248	238	96%
41	146	143	98%
42	106	105	99%
43	73	73	100%
44	47	47	100%
45	38	37	97%
46	22	22	100%
47	10	10	100%
48	9	9	100%
49	4	4	100%
50	3	3	100%
51	0	0	n/a
52	1	1	100%
53	2	2	100%
Total	16,632	10,889	73%

MWRA's data were similar in changes in female prevalence by size. Their 30-35 cm baseline winter flounder yielded 73% female for the population (Table 4), while the average for the post-diversion sample was 85% (Table 5).

Table 4. Winter flounder percentage female by size (total length in cm). MWRA 1991-2000.

Winter Flounder Length (cm)	Winter Flounder Sample Size (n)	Female (n)	% Female
30	210	150	71%
31	233	165	71%
32	268	192	72%
33	237	175	74%
34	244	183	75%
35	189	139	74%
36	152	127	84%
37	117	107	91%
38	92	83	90%
39	62	61	98%
40	64	62	97%
41	46	45	98%
42	37	36	97%
43	20	20	100%
44	6	6	100%
45	4	4	100%
46	3	3	100%
47	3	3	100%
48	2	2	100%
49	2	2	100%
Total	1991	1565	90%

Table 5. Winter flounder percentage female by size (total length in cm). MWRA 2001-2015.

Winter Flounder Length (cm)	Winter Flounder Sample Size (n)	Female (n)	% Female
30	139	116	83%
31	177	139	79%
32	279	231	83%
33	294	248	84%
34	358	294	82%
35	366	335	92%
36	335	301	90%
37	272	245	90%
38	277	260	94%
39	211	205	97%
40	141	137	97%
41	123	120	98%
42	81	81	100%
43	80	79	99%
44	41	41	100%
45	18	18	100%
46	16	16	100%
47	9	9	100%
48	9	8	89%
49	3	3	100%
50	4	4	100%
51	1	1	100%
Total	3234	2891	93%

During the baseline period (Figure 12), the outfall was located in Boston Harbor, and not in Massachusetts Bay. Therefore, Figure 12 is relevant only in comparison to the post-diversion period (Figure 13) to observe any temporal trends in the prevalence of female winter flounder. Ratios of female flounder in the baseline period mostly range between 43-85%, with higher percentage females in the larger size groups. The female percentage of winter flounder increased to a range of 56%-88% in the same cm classes of post-diversion period. Comparing the post-diversion (Figure 13) to the baseline period (Figure 12) indicates that the proportion of female to male winter flounder increased.

Most interesting are the 29 and 30 cm groups, in the <10 and 10<20 mile ranges. Those categories have experienced a 13% increase in female flounder (Figures 12 & 13).

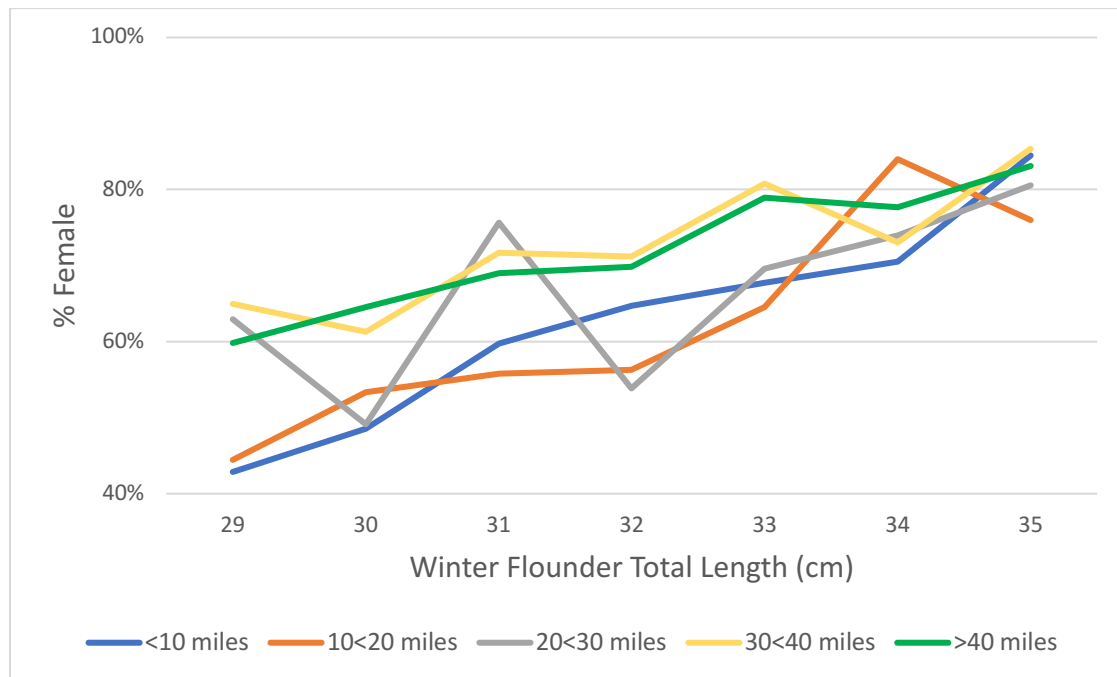


Figure 12. Percentage female winter flounder per cm (total length) based on distance ($\pm$ 0.6 miles) from the future site of the MWRA Mass Bay outfall. NEFSC & DMF sampling 1980-2000.

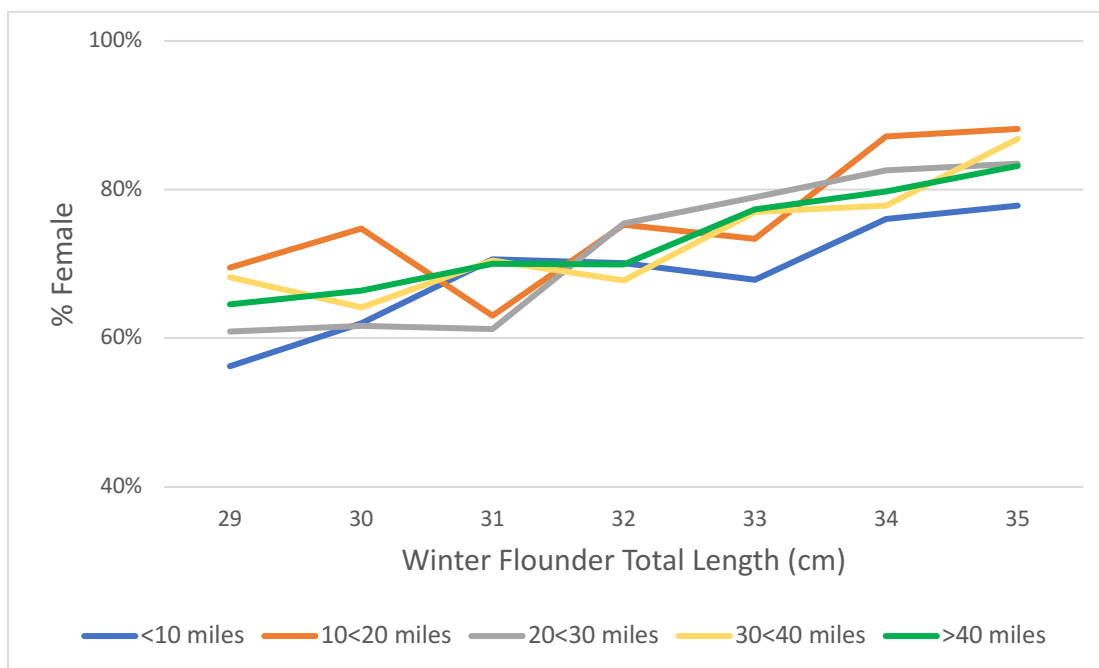


Figure 13. Percentage female winter flounder per cm (total length) based on distance ($\pm$ 0.6 miles) from MWRA Mass Bay outfall. NEFSC & DMF sampling 2001-2017.

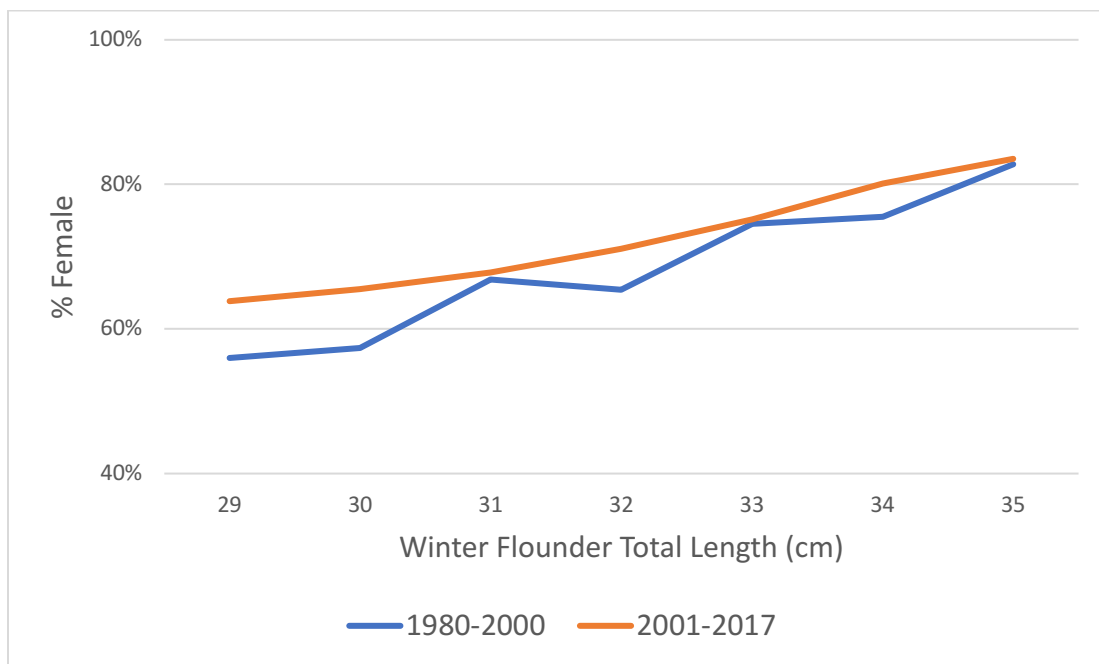


Figure 14. Winter flounder female prevalence by total length NEFSC and DMF 1980-2000 (baseline) and 2001-2017 (post-diversion).

Direct comparison of the baseline and post-diversion periods shows this increase in percentage female within the smaller size groups (Figure 14). Larger sizes were predominantly female throughout all sampled years.

Due to the fact that ≥ 36 cm flounder tend to be female (baseline 92% female, and post-diversion 94% female), MWRA's smaller sampled size categories, 30-35 cm, were used to illustrate baseline and post-diversion trends (Figures 15 & 16). This also allowed comparison with the NEFSC and DMF figures. Baseline and post diversion trends are plotted together for NEFSC & DMF (Figure 17) and MWRA (Figure 18).

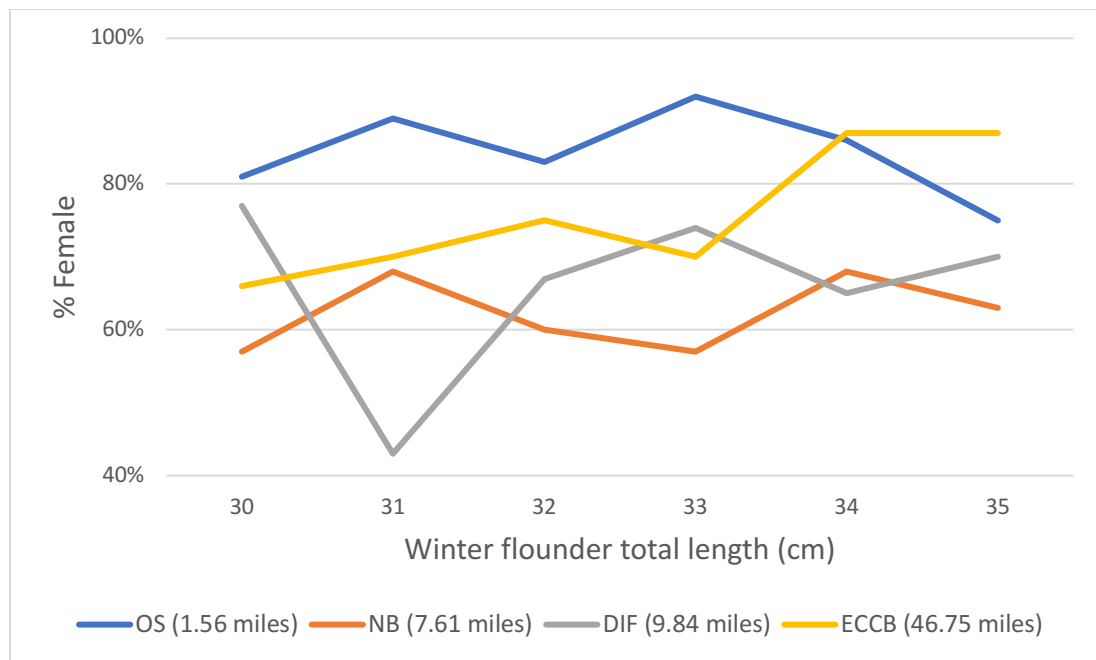


Figure 15. Winter flounder percentage female by length (30-35 cm) relative to distance from outfall (± 0.6 miles). 1991-2000 MWRA.

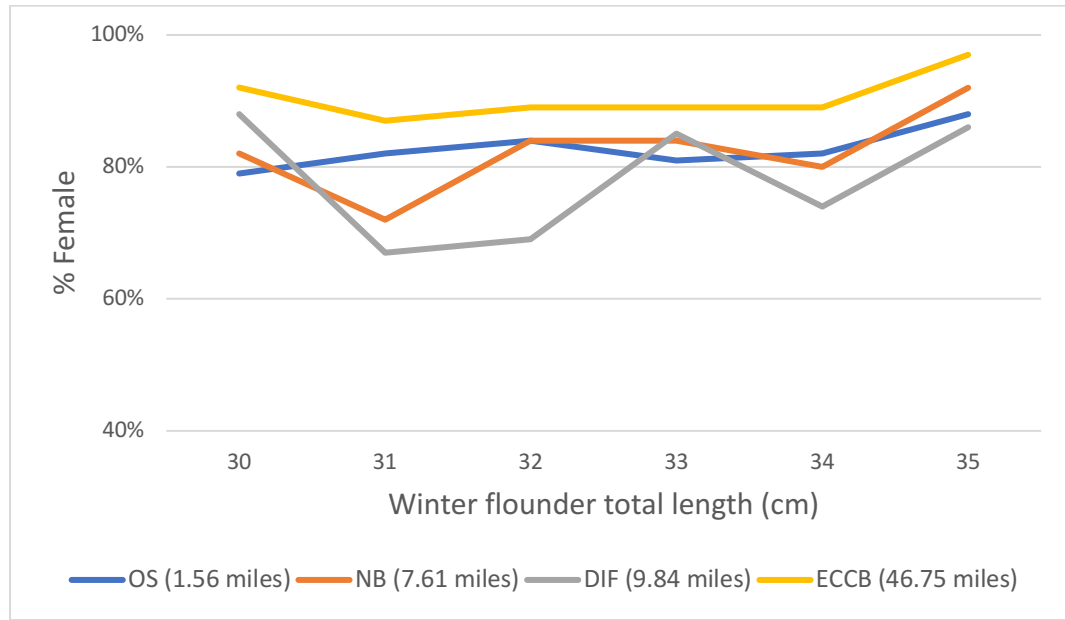


Figure 16. Winter flounder percentage female by length (30-35 cm) relative to distance from outfall (± 0.6 miles). 2001-2015 MWRA.

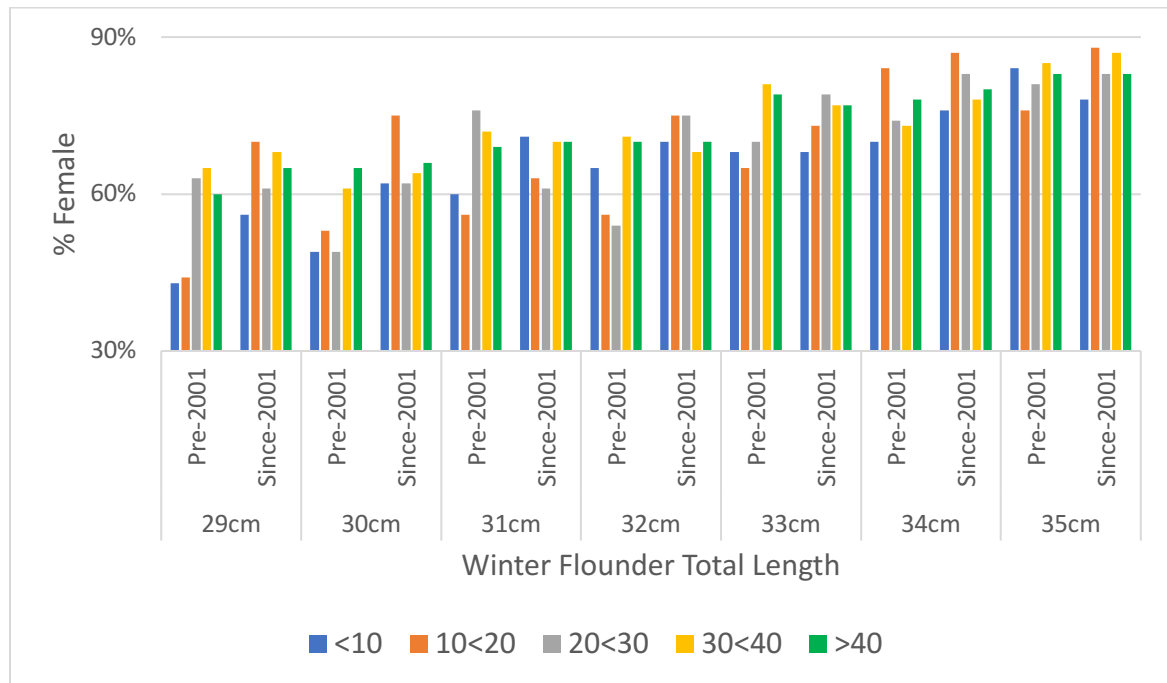


Figure 17. NEFSC & DMF winter flounder percentage female by total length (cm) and distance from Mass Bay outfall. Percentage female by individual size groups of winter flounder, pre-2001 (baseline) and since-2001 (post-diversion), relative to distance (± 0.6 miles) from the Massachusetts Bay outfall.

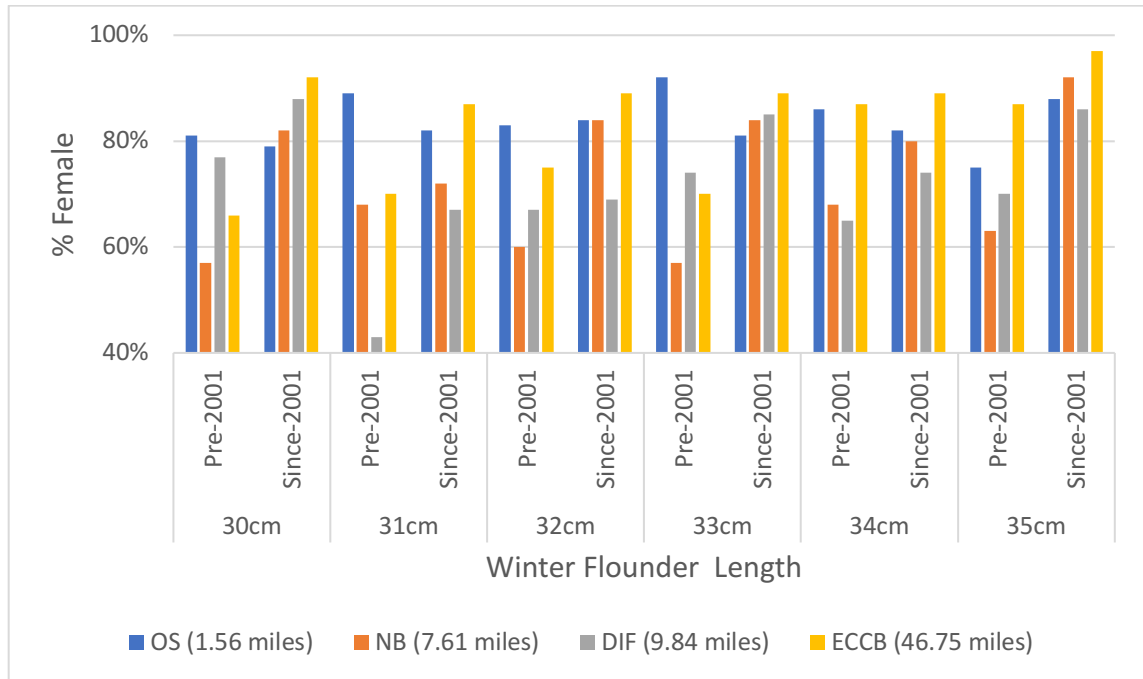


Figure 18. MWRA percentage female by total length (cm) of winter flounder, pre-2001 (baseline) and since-2001 (post-diversion), relative to distance (± 0.6 miles) from the Massachusetts Bay outfall.

Baseline and post-diversion sex data were entered into QGIS for mapping (Figure 19). The maps include MWRA's four sampling locations and the Mass Bay outfall (even though it was not yet active during the baseline period). Female flounder were found all throughout Massachusetts Bay and Cape Cod Bay. During the baseline period, stations ECCB, DIF and NB are all close to anthropogenic sources of contamination. The OS site became a source of contamination after the summer of 2000, after the startup of the outfall. These maps illustrate how winter flounder males and females were scattered throughout the area. Most of these flounder were north of the outfall or in the south, within Cape Cod Bay. As previously mentioned, the smaller size categories (29-30 cm) showed elevated female sex ratios, especially for distances <10 and $10 < 20$ miles from the outfall.

Spawning locations were also mapped out in QGIS (Figure 20), using baseline and post diversion data. The data used in Figure 18 included all size groups sampled by NEFSC and DMF. All sizes were included to provide a better understanding of where winter flounder may have been spawning. Maturity categories ripe, ripe and running, and spent are indicated in the map. This clearly illustrates that winter flounder were spawning offshore, as observed in prior research (Fairchild, 2017). GOM winter flounder are known to spawn inshore during the winter, and head out to deeper waters when they are not spawning. The majority of spawning activities (Figure 20) were likely occurring inshore, but there are many winter flounder in late stages of reproduction (ripe/ ripe and running), as well as those that are spent, that seemed to be spawning offshore.

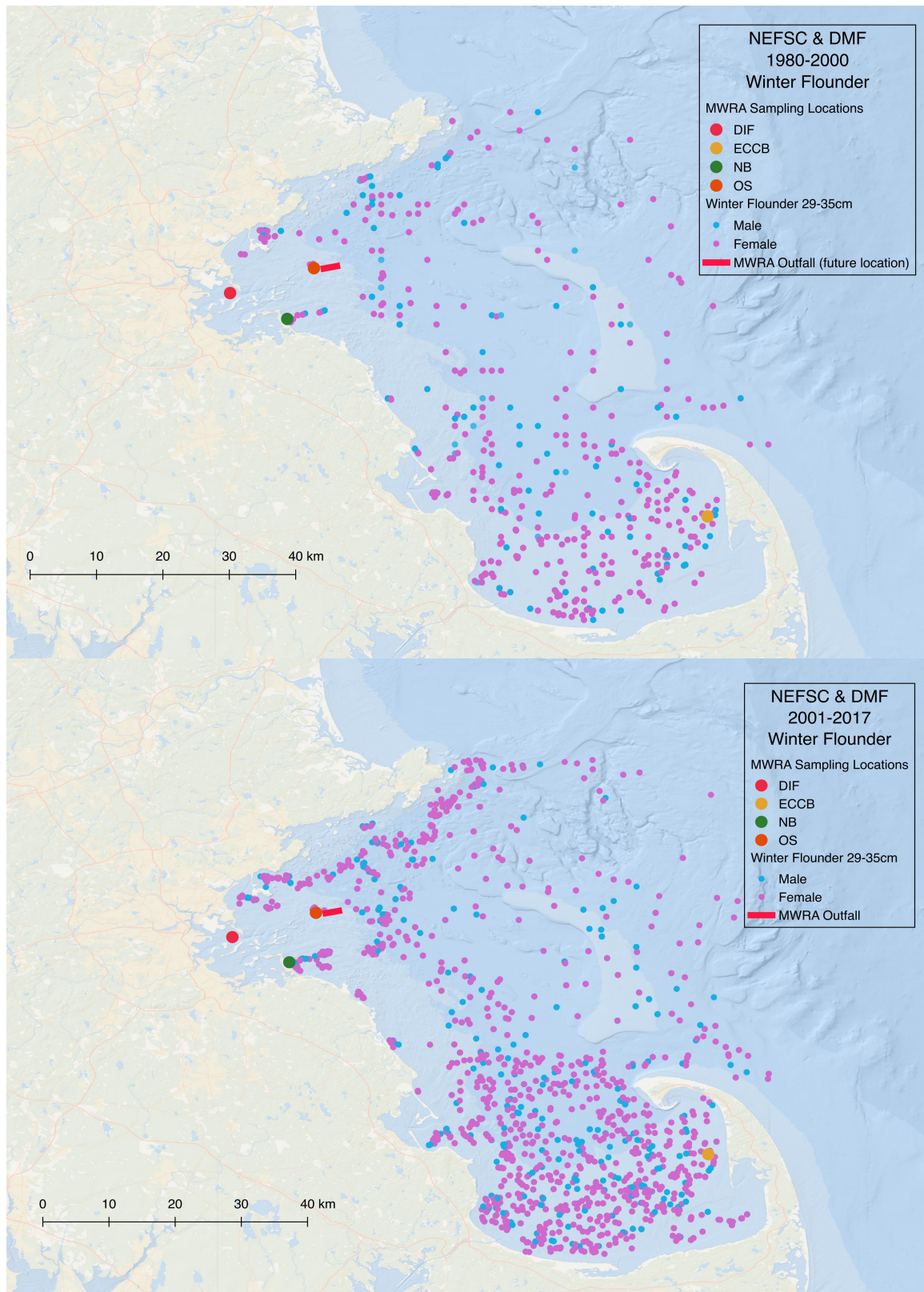


Figure 19. Winter flounder distribution by sex during the baseline and post-diversion periods for NEFSC & DMF sizes 29-35 cm (TL) (sources ESRI, NEFSC, DMF, MWRA).

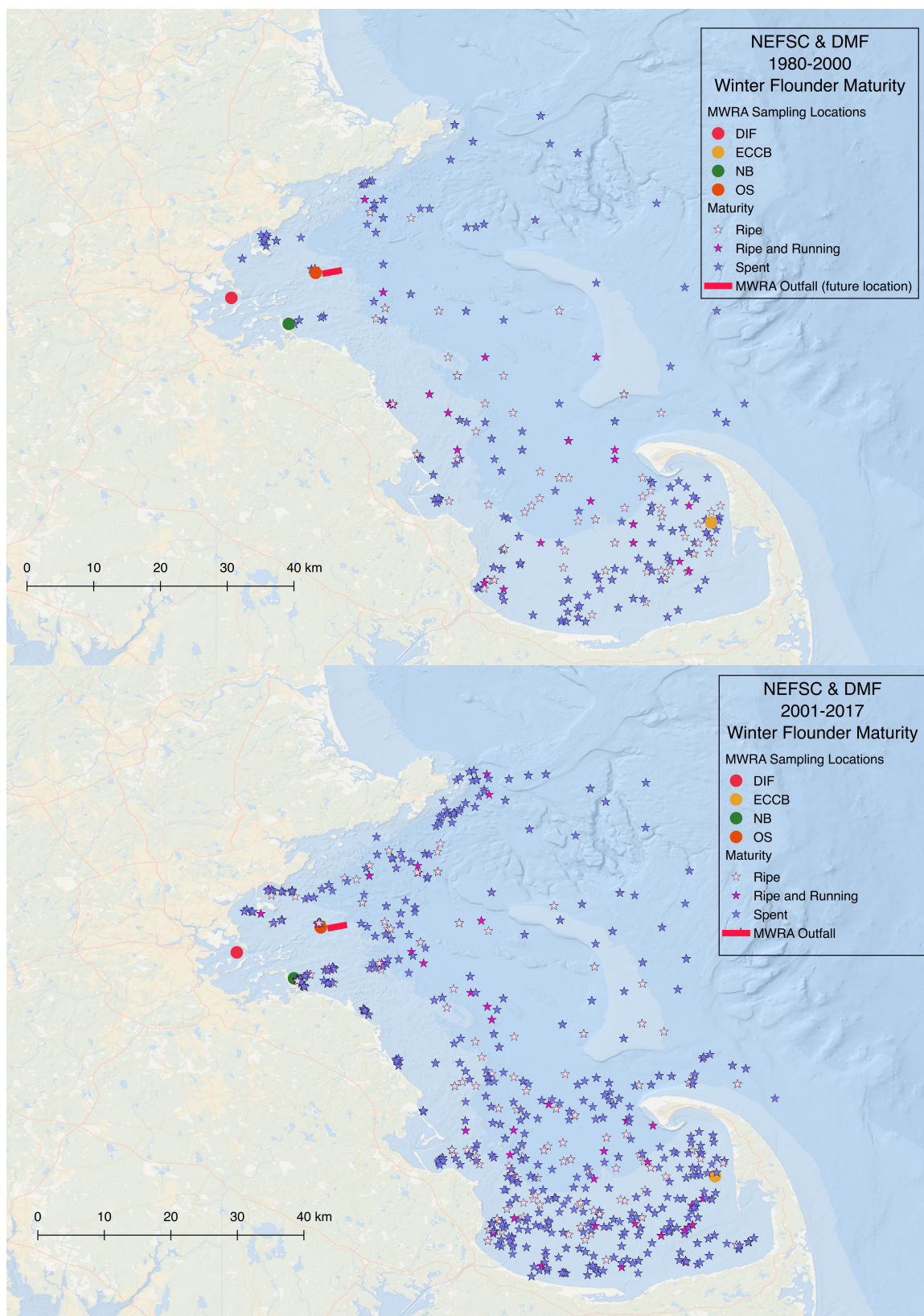


Figure 20. Winter Flounder spawning locations based on maturity data from NEFSC and DMF bottom trawling, baseline and post diversion periods.

Although sample sizes for the post-diversion period are significantly higher than the baseline, the proportion of winter flounder in each of the reproduction stages based on distance was similar during the two periods (Figures 21 & 22). However, there does seem to be a higher proportion of immature flounder <10 miles from the outfall.

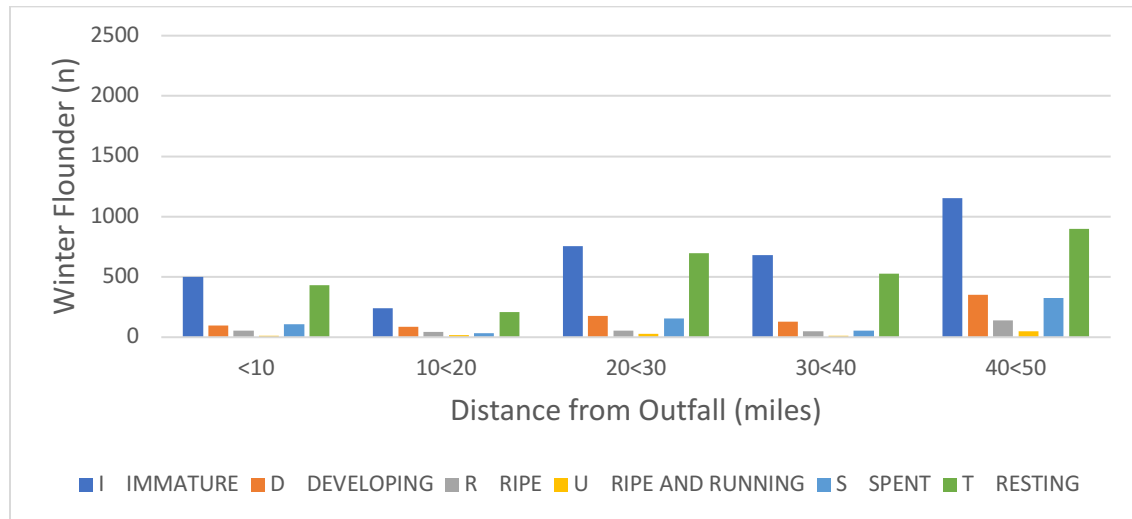


Figure 21. Winter flounder maturity relative to distance from Mass Bay outfall. Baseline, NEFSC & DMF 1980-2000.

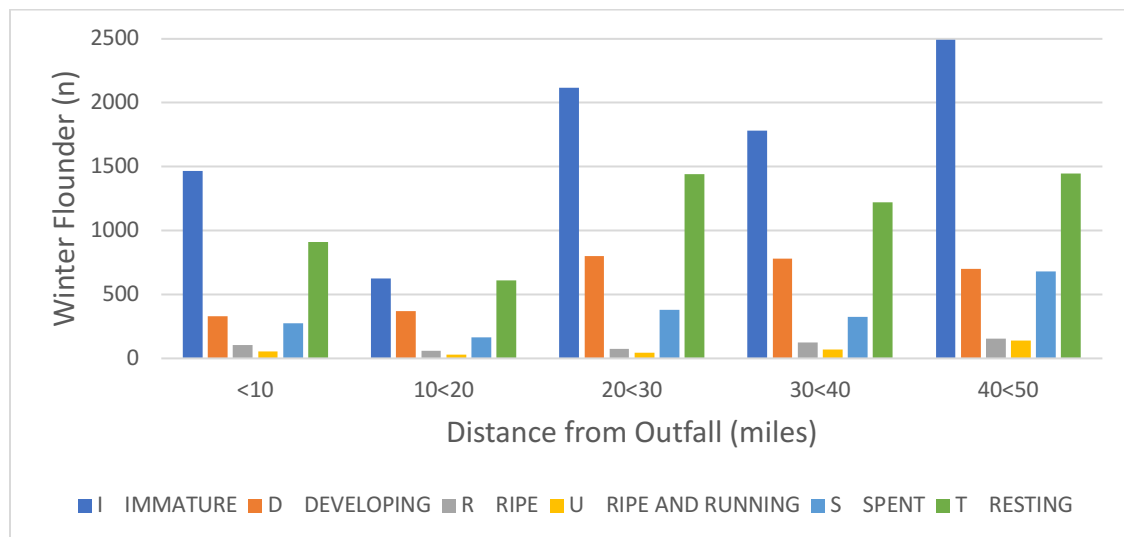


Figure 22. Winter flounder maturity relative to distance from Mass Bay outfall. Post-diversion, NEFSC & DMF 2001-2017.

Chapter IV

Discussion

The samples of fish collected <10 miles and 10-20 miles from the Deer Island Wastewater Treatment Plant (DIWWTP) outfall site have experienced an increase in the proportion of female flounder in the combined post-diversion 2001-2017 samples compared to the baseline period. NEFSC and DMF winter flounder in size groups 29 cm and 30 cm showed a 13% increase in females from the baseline (1980-2000) to the post-diversion periods (2001-2017).

However, the test of the hypothesis was weakened because the sex ratio change could only be examined in the 29-35 cm size classes. Winter flounder smaller than 28 cm tend to be immature, and those larger than 35 cm tend to be female. In addition, the NEFSC and DMF samples were of single fish per cm class, and not total population samples. Sample size were extremely small for individual size groups close to the outfall site.

Improving Understanding of the Effects of APIs and PPCPs

The decline in commercial landings for winter flounder hurts consumers, fishermen, and the economy. Greater resources need to be placed into understanding why an entire stock is in decline, even when overfishing is not occurring.

Moving the outfall from the Deer Island location to the current one in Massachusetts Bay has greatly improved water quality in Boston Harbor. This is also true when comparing effluent discharge in hydrodynamic modeling from the Boston Harbor

outfall to that of the Mass Bay outfall (Geyer, 1999). However, these models do not study the long-term effects of APIs (active pharmaceutical ingredients) and PPCPs (pharmaceuticals and personal care products) on the health of the marine ecosystem. There is no denying that moving the outfall has helped make Boston Harbor cleaner, but the next step is to consider PPCPs in effluent and how that needs to be studied further. Although sampling continues by DMF and NEFSC, they are not specifically targeting the sex ratio composition of GOM winter flounder by performing a randomized population-wide study. There are also not enough sampling points within a 5 miles radius of the outfall.

In order to have a better understanding of APIs (active pharmaceutical ingredients) from the outfall, random sampling should be conducted in Massachusetts Bay, ensuring that the entire tow of winter flounder is included in the study. In addition, sampling needs to be performed closer to the outfall. Currently, NEFSC and DMF protocols sample per cm class, ensuring that at least one fish per cm is collected for each tow. Sampling also needs to be random for the entire tow, and not just by cm.

Similarly, MWRA sampling is limited due to the fact that they only monitor four locations, almost all of which are close to anthropogenic contaminants. They also only include data for winter flounder > 30 cm. This naturally would skew the overall results in favor of females, since female flounder grow larger than males. Sample sizes within size categories 30-35 cm were smaller than the combined NEFSC and DMF samples. From 2001-2015, MWRA sampled 139 flounder at a total length of 30 cm, while the combined NEFSC and DMF sample size was 765 flounder for that same size category. Unlike the

NEFSC and DMF results, MWRA's 30 cm group had a slight decrease in the female prevalence from the baseline to the post-diversion period.

MWRA's OS, or outfall sampling site, is not at the actual outfall location, but 0.91 miles west of the closest outfall diffuser head, and 2.14 miles from the furthest point. Sampling needs to be conducted north, south and east of the outfall to consider currents and the overall water dynamics in the area. In general, not enough locations are sampled close enough to the outfall, by any of the agencies, to allow for a better understanding of sex ratio and outfall effects. In general, there would be a clearer picture of population dynamics if MWRA and NEFSC/DMF standards include all winter flounder sizes as well as random sampling of the entire tow.

In addition, although it is not required by the EPA, sediment sampling conducted by the MWRA should include a study on APIs. Pharmaceuticals may contribute to the deterioration of winter flounder spawning habitats. Lab studies reveal that winter flounder can withstand water quality problems (Maine.gov, 2016). Although the ecological linkages are speculative, the introduction of pharmaceuticals into these habitats may be contributing to the lower commercial seafood landings.

In addition to the above, a special study is necessary to examine winter flounder in the lab, checking for any traces of APIs or reproductive issues. Because winter flounder move from inshore to offshore locations throughout the year, it is difficult to connect their exposure and uptake of APIs to one specific site. However, lab studies would help determine if PPCPs are appearing in flounder, a clear sign of effluent contamination. A tagging study of GOM winter flounder in Massachusetts Bay would provide crucial details about winter flounder movements throughout their lives. It is

important to better understand if exposure to anthropogenic pollutants are mostly occurring in estuaries or near the sewage outfall site.

It is also critical to include pharmaceuticals in drinking water and wastewater quality standards. Although current EPA standards do not include requirements for APIs in wastewater treatment, Massachusetts needs to devote resources into the removal of APIs from effluent before its release into the environment. Otherwise, winter flounder monitoring must include a study into the effects of APIs on winter flounder and other marine species. Moving the location of the Deer Island outfall from Boston Harbor to Massachusetts was a positive first step in improving the overall health of the Harbor. Today, we must go even further and work on these important issues and make them a priority for our government.

Conclusions

The research results verified that proximity to the Deer Island Wastewater Treatment Plant (DIWWTP) outfall site in Massachusetts Bay was associated with an increase in female-biased sex ratios. When all of the data were organized and analyzed, NEFSC and DMF size groups 29 cm and 30 cm revealed a significant increase in female prevalence in the post-diversion sample. APIs in wastewater effluent could be the reason, but this requires further research.

It is clear that none of the agencies are currently working to understand the declining sex ratio of winter flounder. A randomized tow of winter flounder is necessary, which would include all size groups, and better help us in calculating the sex ratio. It is

also critical to include more sampling points close to the outfall, and test for pharmaceuticals in flounder and sediment. Tagging studies might reveal important data about where winter flounder may be exposed to PPCPs.

Winter flounder populations could rebound if enough attention is placed into understanding the many stresses from anthropogenic contaminants. The Earth is a closed system; water, air, and soil are all connected. Pollution in one area of the world spreads to places much further away, with unintended consequences. Will our actions today help or hurt our food resources for tomorrow?

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