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## Investigating the Endocrine Disrupting Potential of the Effluent-Dominated Assabet River

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**INVESTIGATING THE ENDOCRINE DISRUPTING POTENTIAL OF THE  
EFFLUENT-DOMINATED ASSABET RIVER**

A Thesis Presented

By

KASIE M. AUGER

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Massachusetts Amherst in partial fulfillment of the  
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## **DEDICATION**

To My Father, William James Auger Jr., the greatest man I ever knew.

## ACKNOWLEDGEMENTS

I would like to acknowledge all the people in my life who have helped me reach this milestone. I have logged a lot of hours and put in a lot of hard work but none of it would be possible or as meaningful without all those who surround me.

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## ABSTRACT

### INVESTIGATING THE ENDOCRINE DISRUPTING POTENTIAL OF THE EFFLUENT-DOMINATED ASSABET RIVER

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The Assabet River located in eastern Massachusetts receives treated wastewater discharges from four major municipal wastewater treatment plants (WWTPs): Westborough, Marlborough, Hudson and Maynard. In periods of low flow, up to 95% of the Assabet River is wastewater effluent. Chemical analyses have shown that municipal wastewaters can contain estrogenic and dioxin-like compounds. Estrogenic compounds such as the natural estrogen 17 $\beta$ -estradiol (E2), the synthetic estrogen 17 $\alpha$ -ethinyl estradiol (EE2), and the industrial compound nonylphenol (NP) can induce vitellogenin (VTG) and lead to feminization in male fish. CYP1A1-inducing compounds such as the polycyclic aromatic hydrocarbons (PAHs) and polybrominated diphenyl ethers (PBDEs) can produce both overt toxicity and alter reproductive function through the metabolism of natural estrogens. The purpose of the present research is to analyze the estrogenic and CYP1A1-inducing compounds in the Assabet River and their physiological effects on Japanese Medaka (*Oryzias latipes*). I used a bioassay that measures the induction of VTG and CYP1A1 in the livers of male Medaka and report results obtained by the USGS and EPA on analytical measurements of selected compounds.

In the summers of 2010 and 2011 water samples were collected from the Assabet River, its tributaries and the four WWTPs. Male Medaka were exposed to the treatment

samples as well as negative and positive controls. VTG and CYP1A1 induction were measured using real time RT-PCR. Concurrently collected samples from 2010 were analyzed by the USGS for more than 80 organic wastewater contaminants including several estrogenic EDCs and CYP1A1-inducing compounds. The USEPA also analyzed treated wastewater effluent samples collected from the four WWTPs for pharmaceuticals, hormones, nonylphenols and perfluorinated compounds. The bioassay from 2010 and 2011 reveal no statistically significant induction of VTG expression and only one significant induction of CYP1A1 expression. Few compounds were detected by the 2010 USGS and USEPA chemical analyses and the concentrations were low. Taken together the results indicate that VTG and CYP1A1 inducing compounds in the effluent-dominated Assabet River are present at low levels, which may be below the level of detection of the bioassays.

In addition to the biological assay and chemical analysis Geographic Information Systems (GIS) was used to analyze land use/land cover (LU/LC) data in the Assabet River Watershed. Much of the land surrounding the Assabet River is forested but there are several LU/LC types that could negatively impact the water quality. High impact and low impact LU/LC types were differentiated in buffers around the Assabet River and six GIS sites. The composition of each site varies widely in its proportions of high and low impact land cover. The GIS analysis established locations on the Assabet River where water quality is more susceptible to degradation due to the distribution of high impact land use types.

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## CHAPTER 1

# GENE EXPRESSION OF VITELLOGENIN AND CYTOCHROME P4501A1 IN MALE JAPANESE MEDAKA (*ORYZIAS LATIPES*) EXPOSED TO ASSABET RIVER, TRIBUTARY AND EFFLUENT WATER SAMPLES

### Introduction and Background

#### **The Assabet River**

The Assabet River is located in eastern Massachusetts. It begins at the headwaters in Westborough and winds northeast for 31 miles towards Concord. It passes through 19 cities and towns covering 177 square miles with a population of 170,000 residents. Some notable animal species living in or near the river are beavers, rainbow trout and bald eagles. Many local residents use the river as a source of recreation for water sports such as kayaking and canoeing. The river is also home to four major and three minor municipal wastewater treatment plants (WWTPs). These plants hold National Pollution Discharge Elimination System (NPDES) permits to release treated effluent into the river.

Wastewater treatment plants are highly regulated operations, processing millions of gallons of wastewater each day with facilities such as the Westborough WWTP managing 7.68 million gallons per day (mgd) [1]. Effluent discharge was not always as regulated as it is today and as recently as the 1940s factories and municipal WWTPs in mill towns were releasing untreated effluent from factory operations directly into rivers and streams including the Assabet River.

Historically, in the United States, and particularly in New England, rivers have been dammed to power mills. There are nine dams on the Assabet River seven of which are old milldams (Figure 1.1). Dams create pond-like impoundments that slow the flow of the water increasing nutrient concentrations and the growth of native and invasive

plants, which can threaten the health of the river. In addition to damming parts of the river, mills used to dump untreated effluent directly into the river. In the 1940s local residents knew whether the color of a woolen order was yellow, blue or green because the river would turn the same color [2]. Today municipal WWTPs discharge millions of gallons of treated effluent directly into the river. In addition to treated wastewater effluent urbanization also threatens the health of the river.

Although much of the land surrounding the Assabet River is forest, urbanization has negatively impacted the river. The loss of natural flow in the Assabet and its tributaries from dams, ground water withdrawals, sewer systems and the increase in impervious surfaces is profound. This decrease in baseflow means that in dry summers the water flowing in the Assabet River can be upwards of 90% treated effluent [3]. Currently four major municipal WWTPs (Marlborough, Westborough, Hudson and Maynard) are located on or near the Assabet and release treated effluent directly into the river. Treated effluent often contains chemical compounds such as veterinary and human antibiotics, prescription drugs, nonprescription drugs, steroids and hormones [4,5]. These compounds as well as mixtures of these compounds can impact human and environmental health [6,7]. Fortunately, there is a concerted ongoing effort to fight the negative impacts of increased urbanization and ensure the health and safety of the Assabet River.

Many management practices have been implicated on the river for restoration and conservation. Over the past several decades volunteers as well as paid staff from several organizations have come together to measure and record basic water chemistry parameters such as dissolved oxygen, pH and phosphorous. This has resulted in several

grant-supported research and clean up opportunities as well as the creation of The Assabet River National Wildlife Refuge by the U.S. Fish and Wildlife Services and National Wildlife Refuge Systems. The goal of one non-profit organization, OARS Inc., is to elevate the status of the river to an EPA class B river – fishable and swimmable. In cooperation with OARS this study incorporates chemical analysis in conjunction with biological assays to evaluate the estrogenic and dioxin-like activity of water samples collected from the Assabet River, its tributaries and treated wastewater effluent.

### **Endocrine Disruptors and Wastewater Effluent**

A 2012 report by the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) describe endocrine disruptors as,

“An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations [8].”

Endocrine disruptors have been found in surface waters throughout North America and Europe [4,9-12], which can adversely impact aquatic organisms, human health and the environment [13-17]. Some sources of endocrine disrupting compounds are pharmaceuticals and personal care products (PPCPs), industry and agriculture [18,19]. There are two points of entry for compounds into surface waters; point sources and non point sources. Nonpoint sources of pollution are agriculture, mining, roadways and residential development [20]. Point sources of pollution are Concentrated Animal Feeding Operations (CAFOs) [21], Combined Sewer Overflows (CSO) [22-24], and WWTPs [16,17]. In this chapter I will focus on point sources of pollution namely WWTP effluents, and I will discuss nonpoint source pollution in more detail in chapter two.

WWTP effluent is treated wastewater that is released into nearby water bodies and often contains excreted hormones, pharmaceuticals [17,25,26] and industrial chemicals [17,27,28]. These compounds can negatively affect aquatic organisms living in close proximity to effluent outfalls. A study by Canobbio et al. [29] describes how benthic macroinvertebrate communities can suffer when in close proximity to WWTP effluent outfalls and more specifically from CSO events when untreated effluent enters the environment due to large precipitation events. Tetreault and colleagues [30] demonstrated the adverse biological effects of concentrated municipal effluent on Fathead Minnow (*Pimephales promelas*) and Brook Stickleback (*Culaea inconstans*). These effects included changes in kidney and gill morphology, reproduction abnormalities and induction of vitellogenin (VTG) in males. VTG induction is a biomarker of exposure to estrogenic endocrine disrupting compounds (EDCs) and the main focus of the present research.

VTG is an egg yolk precursor protein found at high levels in females and very low levels in males. It is induced by estrogenic compounds, such as the natural estrogens, estrone (E1) and 17- $\beta$  estradiol (E2), and the synthetic estrogen, 17- $\alpha$ -ethinyl-estradiol (EE2) [15,16,31]. Many currently used WWTP processes do not completely remove or break down these estrogens [12], which results in low level (parts per trillion, ppt) concentrations (~5 ng/L) in the environment [16,32-34]. VTG induction can occur when fish are exposed to very low concentrations of E2 and EE2 [35]. Seki et al. [36] demonstrated an LOEC (lowest-observed-effect concentration) for EE2 in Medaka of 0.0639  $\mu\text{g/L}$ , while Kang et al. [37] demonstrated an LOEC for E2 in Medaka of 0.0557  $\mu\text{g/L}$ . In addition to natural and synthetic estrogens, compounds with chemical structures

similar to the natural and synthetic estrogens, such as Bisphenol-A (BPA) [38] and nonylphenol [39], can induce VTG and are referred to as estrogen mimics. In addition to VTG induction, other intersex characteristics in males including the presence of ova in the testes [15,16] and hermaphroditic male fish downstream of WWTPs are biomarkers of exposure to estrogenic compounds [15,40].

A large body of data exists demonstrating the impacts effluent can have on aquatic organisms, human health and the environment. The present research focuses on the gene expression disruption that occurs when fish are exposed to treated effluent. I used Japanese Medaka (*Oryzias latipes*), to examine the impacts associated with exposing organisms to treated effluent and Assabet River water samples.

### **Japanese Medaka (*Oryzias latipes*) a Model Organism**

For this research project I used the model laboratory organism, Japanese Medaka (*Oryzias latipes*), a freshwater fish species. Medaka are naturally tolerant to low water temperatures because they are native to temperate East Asian countries mainly Japan, China, Taiwan, and Korea. Due to their temperate habitat Medaka can withstand temperatures ranging from 4°C to 40°C [41], which makes them a good model to represent fish in the north Atlantic region of the US. They are hardy aquatic organisms because they can withstand a pH range of 6.8-8.0 and a conductivity range of 500-700 µS. In addition, Medaka are ideal laboratory organisms because their small size allows for the housing of several hundred to several thousand individuals in one laboratory.

Medaka are a model aquatic organism widely used for toxicological testing. Medaka are used for internationally standardized toxicity tests such as acute, early life stage and life cycle toxicity tests. Acute toxicity tests generally expose 7 individuals to

test chemicals for 96 hours to determine the lethal dose of test chemicals. In early life stage toxicity tests researchers look for changes in growth and behavior in egg, larval and juvenile life stages. Japanese Medaka mature in a relatively short time (~8 weeks), which aids in life cycle toxicity testing during which the researcher can look for changes in both the parent and offspring in as little as four months [42]. Medaka are also used in tests for endocrine disruptors, which is the focus of the present research.

Medaka are used in laboratory studies to test for the presence of endocrine disruptors because the male Medaka is a sensitive fish model and exposure to exogenous endocrine disruptors causes measurable physiological changes. One of these changes is the induction of VTG an egg yolk precursor protein specific to female Japanese Medaka and a second characteristic is the development of primary oocytes in the testes of male fish [36,37,43,44]. This study will focus on the induction of VTG in the livers of male Medaka exposed to endocrine disruptors. I performed a biological assay using Japanese Medaka and combined that information with chemical analysis data to describe the endocrine disrupting potential of the effluent-dominated Assabet River.

### **Biological Assays and Chemical Analyses**

Surface water is often a complex mixture of water, particulates organic and inorganic compounds originating from natural and anthropogenic sources. Large amounts of water quality data have been recorded and managed by regulating bodies, such as state and federal governments, and non-governmental bodies, such as academic institutions and non-profit organizations. There are different methods of obtaining and interpreting water quality data including chemical analysis and biological assays. For this study more

emphasis was placed on interpreting the biological analysis data but interpretations from chemical analyses will also be described.

Biological assays measure physiological, behavioral and generational changes in biological organisms that would be impossible with the use of chemical analysis alone. Bioassays are often more informative than chemical analyses because they can tell us the effects mixtures of compounds have on biological organisms as opposed to just the presence and concentration of individual compounds in water. Studying the effects of chemical mixtures is more complicated than studying the effects of individual chemicals because mixtures can have additive, synergistic or negating effects. An additive effect is when the total effect of two or more substances or actions used in combination is the same as the sum of the individual effects or actions. A synergistic effect is when the total effect of two or more substances or actions used in combination is greater than the sum of the individual effects or actions [45]. Bioassays are often used in conjunction with chemical analysis and choosing the right bioassay is integral to the success of any research project.

There are several types of bioassays used for toxicity testing. Acute toxicity tests expose organisms for 96 hours or less and chronic toxicity tests can last for weeks, months or even years. Acute toxicity tests can use a wide range of life stages from embryonic to adult but often have to choose one life stage. Chronic toxicity tests can start with a wide range of life stages but unlike acute testing can measure changes over an individual's lifetime as well as generational changes. Acute toxicity tests are valuable because they use fewer resources than chronic exposures and can reveal robust responses in a short time. Chronic toxicity tests are valuable because many test species have short

maturation times and responses of several generations can be observed in a relatively short time, weeks or months. Chronic low-level toxicity tests are becoming increasingly valuable to scientists who want to see the effects experienced by wild populations exposed to environmentally relevant low levels of compounds [16]. For the present research I performed an acute toxicity test with a 72-hour exposure to measure estrogenic activity of water samples taken from the Assabet River, its tributaries and WWTP effluent.

The bioassay for this study used medaka to measure estrogenic EDC and dioxin-like activity in the water samples collected from the Assabet River watershed. In order to measure estrogenic and dioxin-like activity I exposed male medaka (N=5) to five Assabet River and tributary water samples and measured hepatic VTG and CYP1A1 expression. I chose to measure these genes because they are biomarkers of exposure to xenobiotics and I chose the liver because it is the major tissue involved in xenobiotic metabolism.

The VTG bioassay is frequently used to measure estrogenic activity induced by environmental water samples. VTG is a sensitive biomarker used in several fish models because gene expression can increase dramatically when fish are exposed to estrogenic EDCs. Figure 1.2 demonstrates how VTG induction occurs in male Medaka exposed to an exogenous synthetic estrogen. The exogenous estrogen enters the male body and reaches the liver through the blood stream. In the liver the exogenous estrogen binds the estrogen receptor (ER) and forms the hormone estrogen receptor complex (Figure 1.3). This complex must dimerize and bind the DNA at the estrogen response element. This binding activates transcription of mRNA, which is translated into protein. Natural estrogens (e.g. E2), synthetic estrogens (e.g. EE2) and estrogen mimics (e.g. BPA) bind

the ER causing the cascade of events that leads to translation of the VTG protein. VTG induction is often measured in males because expression is naturally low or non-existent. Measuring VTG induction in females is less sensitive than in males because increases in expression above their normally high levels is often only seen when females are acutely exposed to extremely high concentrations of estrogens and anti-androgens or chronically exposed to low concentrations [46]. One can also measure reduced VTG expression in females exposed to androgens. I chose to measure VTG expression in male Medaka because induction of VTG is a sensitive biomarker of exposure to estrogenic endocrine disruptors. Measuring increases in male hepatic VTG expression only reveals the activity of compounds that bind the estrogen receptor, which is why I chose to measure a second gene. I measured the expression of CYP1A1 to detect the presence of xenobiotics that bind the aryl hydrocarbon receptor (AhR).

CYP1A1 is a member of the cytochrome P450 gene family. P450s are responsible for the detoxification of xenobiotics and steroid hormones, which induces their expression [45]. The CYP1A1 gene is activated through binding of the AhR. A wide range of compounds, including PAHs (polycyclic aromatic hydrocarbons), such as benzo  $\alpha$ -pyrene, and dioxin-like chemicals, such as TCDD (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin), bind the AhR and induce CYP1A1 expression. The binding of PAHs and TCDD can also lead to antiestrogenic activity, as the induced P450s result in increased catabolism and excretion of many estrogens [47]. The opposite effect, increased estrogenic activity, could occur when the natural rate of endogenous endocrine metabolism by P450s is inhibited by their metabolism of xenobiotics [45]. Measuring changes in CYP1A1 expression can be a useful tool but the nature of CYP1A1 makes

interpretation of the results difficult. For this reason it is beneficial to combine chemical analyses and biological assays to measure the presence of aquatic pollutants and the physiological responses in aquatic organisms.

The USGS and USEPA analyzed water samples collected from the Assabet River in 2010 for pharmaceuticals, hormones and other organic wastewater contaminants (OWCs). The USGS analyzed almost 100 Organic Wastewater Contaminants (OWCs), pharmaceuticals and hormones in the water samples collected from the Assabet River and its tributaries [48]. The USEPA analyzed more than 50 pharmaceuticals and hormones in effluent samples collected from the four major WWTPs that release treated effluent into the river [49]. I present the results of the analyses conducted by the USGS and USEPA..

## **Materials and Methods**

### **Animal Rearing and Housing**

Male and female Japanese Medaka were housed and reared together in a recirculating stand-alone flow-through system (Aquatic Habitats, Apopka, FL, USA). All fish were maintained at a constant water temperature of 25°C, a light:dark photoperiod of 16:8, a pH range of 6.8-8.0 and conductivity range of 500-700 µS. Water quality parameters were checked twice a day using the YSI 556 Multiprobe System (YSI INC., Yellow Springs, OH, USA). Medaka were fed twice per day with ~ 0.5 g ground Deli Flake dry food (Brine Shrimp Direct, Ogden, UT, USA). Medaka were fed live brine shrimp hatched from cysts (Brine Shrimp Direct, Ogden, UT, USA) every other day.

### **Field Sample Collection**

In 2010 water samples were collected from five locations along the Assabet River and its tributaries over three days in early August (Figure 1.4). The locations and dates of

collection for these five water samples are: Coles Brook on August 2<sup>nd</sup>, A1 Impound Westboro and RT 9 Westboro on August 3<sup>rd</sup> and Sudbury Rd. Stow and Fort Pond Brook on August 4<sup>th</sup>. The Coles Brook and Fort Pond Brook water samples were collected from tributaries of the Assabet River. I collected water samples with the help of two members of the USGS, Marc Zimmerman and John Colman. Water samples were collected using three 1-L amber bottles for a total of three liters of each water sample. To collect the samples I wore chest waders and walked slowly, so as not to disturb the sediment, into the river until the water level reached my chest. I rinsed the amber bottles three times with river water before collecting each sample. The water samples were kept on ice for several hours during field collections and then placed in fridges at the USGS water science center in Northborough. On August 4<sup>th</sup> the water samples were transferred in coolers to the aquatic toxicology laboratory at UMass Amherst where they were stored at 4°C until the beginning of the exposure experiments on August 8<sup>th</sup>.

In late August 2011 the USEPA New England Regional Laboratory (NERL) in Chelmsford, MA collected nine water samples from the Assabet River and WWTPs. Four of these water samples were collected directly from effluent outfalls with permission from the WWTP operators and five instream water samples were collected from the Assabet River (Figure 1.4). These nine water samples collected around August 22<sup>nd</sup> are: Reference Site, Hudson, Marlboro, Westboro and Maynard effluents and Hudson, Marlboro, Westboro and Maynard Instream. On August 27<sup>th</sup> water samples were transferred on ice from Chelmsford to the aquatic toxicology laboratory and stored at 4°C until the beginning of the exposure experiments on August 31<sup>st</sup>.

## Exposures and Dissections

Exposure experiments were conducted in the aquatic toxicology laboratory at the University of Massachusetts Amherst and a picture from the exposure experiment conducted in 2011 can be seen in Figure 1.5. To begin the experiment 3-Liter plastic tanks were filled with 1000mL of experimental water that had been allowed to reach laboratory room temperature of 22°C. Both experiments included a negative control of fish rearing laboratory water and a positive control of laboratory water plus a natural or synthetic estrogen. The positive control in 2010 was 1nM E2 (272 ng/L 17β-estradiol) and the positive control in 2011 was 16.8 pM EE2 (5 ng/L 17α-ethinylestradiol). The hatch dates for the fish used in the 2010 experiment ranged from July 13<sup>th</sup>, 2009 to July 25<sup>th</sup>, 2009, therefore the fish were almost thirteen months old. Five male fish were placed into each tank for a period of 72 hours. An 80% static renewal was performed at 24 and 48 hours. In 2010 the experiment started on August 8<sup>th</sup> and ended on August 11<sup>th</sup> and in 2011 exposures began on August 31<sup>st</sup> and finished on September 3<sup>rd</sup>. Fish were fed 0.1 g of flake food twice daily. After 72 hours the fish were anesthetized in buffered 0.5% tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA). The fish were removed from the MS-222 with forceps and placed onto one half of a Kimwipe in the dissector's hand. The other half of the Kimwipe was folded over and used to gently dab the excess water from the fish's body. The fish were placed into a plastic weigh boat and weighed using an analytical balance (Tables 1.1 and 1.2). Fish were then sacrificed by severing the spinal cord with sharp dissecting scissors. The livers were carefully dissected and stored in 1mL TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH) at -20°C until RNA isolation.

## **RNA Isolation and Preparation**

The livers of male Japanese Medaka were stored in 1 mL of TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH) and homogenized with 5mm stainless steel beads (QIAGEN, Valencia, CA, USA) in the Tissue Lyser II (QIAGEN, Valencia, CA). The RNA was isolated using a modified single-step phenol-chloroform extraction method [50]. The quality and quantity of the RNA samples were measured using 2  $\mu$ L of sample on the NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) (Tables 1.3 and 1.4). RNA quality was measured by the 260/280 absorbance ratio and RNA quantity of the stock solutions were recorded. The stock RNA samples were diluted in RNase-free water to obtain the experimental RNA solution at a concentration of 0.1  $\mu$ g/ $\mu$ L. Isolated liver RNA samples were stored at -20°C until real time RT-PCR was performed.

## **Real Time Reverse Transcriptase Polymerase Chain Reaction (Real Time RT-PCR)**

Real Time RT-PCR was performed using the Roche LightCycler 1.5 capillary system (Roche, Indianapolis, IN, USA). A master mix was prepared with the QIAGEN One-Step RT-PCT kit (QIAGEN, Valencia, CA, USA) and distributed to individual capillaries matching the appropriate number of reactions for each PCR run. In addition, 0.75  $\mu$ L of sample RNA (0.1  $\mu$ g/ $\mu$ L), SYBR Green dye (SYBR Green, Molecular Probes Inc, Carlsbad, CA, USA) and primers were added to each individual reaction. Primers (Table 1.5) and program settings were obtained from Moffatt et al. 2010 [51]. VTG, CYP1A1, and the housekeeping gene L7 were quantified for every sample. A standard curve made from a tenfold serial dilution of unexposed female Japanese Medaka liver

was imported into each real time RT-PCR run and expression levels were quantified by the Roche software using the second derivative maximum method.

### **Data Analysis**

Raw expression data for VTG and CYP1A1, the genes of interest, were normalized to the housekeeping gene L7. These normalized relative quantification values and were analyzed by the GraphPad Prism v.3.02 software (San Diego, CA, USA). For the statistical analysis one-way ANOVAs were used to compare exposure groups and post hoc tests were used where necessary.

## **Results**

### **Biological Analyses**

Hepatic VTG and CYP1A1 expression levels were measured in male Medaka (n = 5) exposed to Assabet River water samples in 2010 and 2011. In addition to Assabet River samples fish were exposed to tributary samples in 2010 and effluent samples in 2011. Exposures for both years included positive and negative controls. A VTG positive control was used in both years while a CYP1A1 positive control was not used in either year. In 2010 the positive control was 1 nM E2 and in 2011 the positive control was 16.8 pM EE2. These positive controls were used because they induce VTG [16,51]. In November 2011 we conducted a separate experiment exposing fish to a 1 mg/L  $\beta$ NF to use as the positive control for CYP1A1. Although the CYP1A1 positive control exposure was not conducted at the same times as the other exposures we included this positive control in the analyses and on the graphs for comparison. An assay demonstration can be seen in Figure 1.6 and 1.7.

## **Estrogenic Activity in 2010 and 2011**

No statistically significant induction of VTG in the livers of male Japanese Medaka exposed to Assabet River and tributary water samples was observed in either experiment from 2010 or 2011 (Figures 1.8 and 1.9). For the 2010 exposure experiment a one-way ANOVA including all groups was significant ( $P < 0.001$ ), and post hoc t-tests showed hepatic VTG expression in fish exposed to the 1 nM E2 positive control was significantly higher than VTG expression in all other groups. Exposure to E2 induced VTG greater than 22 thousand fold. No statistically significant differences in hepatic VTG expression of the fish exposed to Assabet River and tributary water samples were observed when the one-way ANOVA excluded the E2 positive control.

As in 2010, none of the water samples collected in 2011 induced statistically significant levels of hepatic VTG expression. A one-way ANOVA including all groups showed no significant effect of the 16.8 pM EE2 positive control, which is significantly lower than our standard positive control of 1 nM E2 used in 2010. We chose the 16.8 pM (5 ng/L) positive control because it was the positive control used in a collaboration research project with the EPA exposing fathead minnow (*Pimephales promelas*) larvae to effluent samples. But, as can be seen in Figure 1.9, exposure to 16.8 pM EE2 did not induce VTG expression in our male Medaka. Despite the lack of VTG induction in the fish exposed to 16.8 pM EE2 we think the assay was successful. A positive E2 control with a concentration of 1 nM (272 ng/L) is not the lower limit of our assay and we have shown previously (data not shown) that both 10 pM (3 ng/L) and 100 pM (27 ng/L) E2 induces VTG in the livers of male Medaka [51] however, VTG induction from a short term assay using such low concentrations is far less robust. In future experiments we will

include a series of 10 pM, 100 pM and 1 nM E2 concentrations as controls. Our results show that the limited number of samples collected from the Assabet River, tributaries and effluents in the summers of 2010 and 2011 reveal no statistically significant levels of VTG induction in the livers of male Japanese Medaka.

### **CYP1A1 Activity in 2010 and 2011**

None of the water samples collected from the Assabet River or its tributaries in 2010 induced statistically significant CYP1A1 expression levels in the livers of male Medaka (Figure 1.10). A one-way ANOVA of all groups including the positive control group was significant ( $P < 0.001$ ). Therefore, I conducted a one-way ANOVA excluding the positive control and no significant differences were found. Exposure to the 1 mg/L  $\beta$ NF positive control induced CYP1A1 expression roughly 12 fold higher than the laboratory water negative control. Also note the high degree of variability of CYP1A1 expression in the livers of male Medaka exposed to the Fort Pond Brook water sample. This high level of variability is due to the high relative expression of one fish, which could not be excluded. The fish was not considered an outlier because the high value was less than the mean plus twice the standard deviation.

The exposure experiment conducted in 2011 revealed that one water sample, Hudson Instream, significantly induced hepatic CYP1A1 expression (Figure 1.11). The positive control for this exposure experiment was not as robust as we had anticipated. The mean hepatic CYP1A1 expression levels of the fish exposed to the 1 mg/L *beta*-Naphthoflavone ( $\beta$ NF) positive control (0.001268 relative fluorescent units, RFU) was lower than the mean of the fish exposed to the laboratory water negative control (0.001741 RFU). The positive control exposure took place in November 2011 whereas

the other exposures took place in September 2011. Therefore, I concluded that the hepatic CYP1A1 expression levels of all our fish in 2011 were elevated compared to 2010. I believe that if I had exposed the Medaka to the positive control in November at the same time as the other exposures I would have observed increased expression levels above the high background of the negative control. With this understanding I conducted a one-way ANOVA to determine if there was any significant effect of treatment despite the increased overall CYP1A1 levels. The ANOVA revealed a significant effect with an F obtained value of 4.56 and F critical value of 2.05. I then conducted post hoc tests to determine which groups differed from the laboratory water. I limited my analysis to those groups with means above the laboratory water negative control standard error of the mean bar. I compared the negative control CYP1A1 levels to Hudson Effluent, Maynard Effluent and Hudson Instream water samples. A two-tailed t-test revealed that the values for the Hudson and Maynard effluents were not statistically above those of the negative control. In contrast, the comparison between fish exposed to lab water and fish exposed to Hudson Instream water revealed that water collected near the Hudson WWTP significantly induced hepatic CYP1A1 expression. This was determined with a two-tailed t-test with a Bonferroni correction for the three comparisons.

### **Chemical Analyses**

Chemical analyses of Assabet River, tributary and effluent water samples were performed by the USGS and the USEPA in 2010. The USGS collected water samples from the five locations marked with a red star in Figure 1.4, and the USEPA collected water samples from the four major WWTP effluent outfalls with the help of the operations managers at the facilities shown on Figures 1.1 and 1.4. The USGS analyzed

Assabet River and tributary water samples for organic wastewater contaminants (OWCs), dissolved organic carbon (DOC), nutrients, pharmaceuticals and hormones (Tables 1.6 and 1.7). Analyses of OWCs, DOC, nutrients and pharmaceuticals were performed at the Water Science Center located in Northborough, MA and hormones were analyzed by AXYS Analytical Services Ltd. The EPA analyzed effluent water samples for pharmaceuticals, steroids/hormones, nonylphenols (NPs) and perfluorinated compounds (PFCs) using the EPA National Effluent Study (NES) protocols. A subset of their findings is presented in this thesis (Tables 1.8 and 1.9).

## **USGS**

The USGS analyzed more than 80 compounds in each of the 11 water samples collected from the five locations in 2010 (Table 1.6). These 80 compounds represent a wide range of pollutants commonly found in aquatic ecosystems. These pollutants have varied classifications, uses, sources, and chemical characteristics. Some general classifications are nutrients, pesticides, polycyclic aromatic hydrocarbons (PAHs), volatile organic carbons (VOCs), PPCPs and polybrominated diphenyl ethers (PBDEs). The uses for some of these compounds include plasticizers, dyes, medications, cosmetics, cleaning products and flame-retardants. A major source of these pollutants is industrial and commercial manufacturing processes. In addition, agricultural practices, pharmaceutical use and consumer products used in every day life are also sources of these pollutants.

### **Analytes Excluding Hormones**

The total number of detects across all categories not including nutrients is 149 (highlighted in yellow in Table 1.6). Thirty-nine total compounds were detected

representing a wide array of classifications including, PAHs, VOCs, PPCPs and pesticides described in Table 1.10. Cholesterol was detected in every sample and DEET was detected in all samples except the two RT 9 Westboro samples. Bisphenol A, a weak estrogenic compound, was found at all locations except Sudbury Rd. Stow. Beta-Sitosterol was detected nine times and Isophorone, Bisphenol A and  $\beta$ -Stigmastanol were each detected seven times. Benzo[a]pyrene, Phenanthrene, Phenol, Naphthalene, Triclosan, 4-Tert-Octylphenol Monoethoxylate and 4-Nonylphenol Monoethoxylate were each detected one time. The highest concentration detected was Bromoform at 22.3  $\mu\text{g/L}$  from the second RT 9 Westboro sample, and the lowest was Indole at an estimated concentration of E0.003  $\mu\text{g/L}$  from the second AI Impound Westboro sample. The number of detects for a single sample range from five for the Coles Brook Replicate sample to 25 for the second Sudbury Rd. Stow sample. The number of detections varied between the two collection dates. The difference in the number of detections between two samples taken from the same location varied from two to nine. In four of the five locations the number of detects was greater on the first day. The one location where detections were greater on the second day was Sudbury Rd, which had 16 detects in the first sample and 25 in the second.

Eleven of the 39 compounds detected are on the ATSDR 2011 Substance Priority List ranging in priority from #8, Benzo[a]pyrene, to #267, Metolachlor. Eleven of the 39 total compounds detected are on the EPA 126 Priority Pollutant List ranging in priority from #27 for 1,4-Dichlorobenzene to #85 for Tetrachloroethylene (PERC). Of the 39 compounds detected, eleven compounds were on both priority lists (Table 1.11).

## **Hormones**

AXYS Analytical Services Ltd. analyzed Assabet River and tributary water samples collected in 2010 for 17 hormones or their metabolites (Table 1.7). This list of 17 hormones consists of natural and synthetic hormones, one medication and a hormone intermediate described in Table 1.12. Three of the hormones are estrogens found in equines, one is a medication used for alopecia, which has antiandrogenic properties and one is a metabolic intermediate. Of the six endogenous hormones listed three are estrogens, two are androgens and one is a progestogen. Of the six synthetic hormones listed two are synthetic estrogens and four are synthetic progestogens.

Overall four hormones were detected and the total number of detects was 11. Androstenedione, an intermediate for the production of testosterone, estrone and estradiol, was detected in seven samples representing all locations. Androsterone a weak endogenous androgen was detected in two samples, one from Fort Pond Brook and one from Coles Brook. Estrone a natural estrogen was detected in one sample from Fort Pond Brook and Testosterone a natural androgen was detected in one sample from RT 9 Westboro. The detection concentrations ranged from 0.45 ng/L for Androstenedione in the first A1 Impound Westboro sample to 305 ng/L for Androsterone in the second Fort Pond Brook sample. Most of the samples had at least one detection except for the second Sudbury Rd. Stow sample and the first Coles Brook sample, which had no detections. Three of the samples had multiple detections. The second RT 9 Westboro sample had two detects, Androstenedione (0.935 ng/L) and Testosterone (0.686 ng/L). The second Fort Pond Brook sample had two detects, Androstenedione (0.76 ng/L) and Androsterone (305

ng/L). The second Coles Brook sample also had two detects, Androstenedione (0.652 ng/L) and Androsterone (194 ng/L).

## **USEPA**

In 2010 the EPA collected water samples from the four major WWTPs on the Assabet River. Using the EPA National Effluent Study (NES) protocols they analyzed the effluent samples for 54 pharmaceuticals, 8 steroids/hormones, 16 nonylphenols (NPs) and 14 perfluorinated compounds (PFCs).

### **Pharmaceuticals**

Twenty-nine of the 54 pharmaceuticals analyzed were detected and organized into seven categories based on their broad mode of action (Table 1.8). These seven categories including the number of compounds in each are: h2 anti-histamine (2), antimicrobial (2), lipid modifier (2), bronchodilator (1), anti-inflammatory (2), anti-hypertensive (7), neurotransmitter modulator (12) as well as one inactive metabolite (Ibuprofen-2-hydroxy). Of the 116 data points there were 73 detections and 42 were not detected above the reporting limit. All broad mode of action categories were represented. Every compound was detected at least one time. Nine of the compounds were found in every sample (Trimethoprim, Triamterene, Desmethyl-diltiazem, Diltiazem, Verapamil, Metoprolol, Carbamazepine, Atenolol and Oxycodone). Eight of the compounds were found in only one sample (Theophylline, Ibuprofen-2-hydroxy, Acetaminophen, Norverapamil, Amitriptyline, Sertraline, Amphetamine, Hydrocodone). Every effluent sample had several detects with Maynard having the most detects (26) and Westborough having the least detects (10). The concentrations of detections varied widely from 1 ng/L (Amitriptyline in the Marlboro west WWTP) to 1663 ng/L (Atenolol in Maynard).

Maynard effluent had the largest number of detects above 1000 ng/L (3) including the largest detection concentration of 1663 ng/L. Six of the data points are near or above the 1µg/L Food and Drug Administration (FDA) categorical exclusion value highlighted in yellow in Table 1. 8. A categorical exclusion value is,

*“Categorical exclusion means a category of actions which do not individually or cumulatively have a significant effect on the human environment and which have been found to have no such effect in procedures adopted by a Federal agency in implementation of these regulations (§1507.3) and for which, therefore, neither an environmental assessment nor an environmental impact statement is required. An agency may decide in its procedures or otherwise, to prepare environmental assessments for the reasons stated in §1508.9 even though it is not required to do so. Any procedures under this section shall provide for extraordinary circumstances in which a normally excluded action may have a significant environmental effect [52].”*

This means that neither an Environmental Assessment (EA) nor an Environmental Impact Statement (EIS) needs to be submitted to the federal government because of these high detections. The six detections that are near or above the FDA categorical exclusion value represent three effluent samples, five compounds and four broad mode of action classes. The Westborough effluent sample had one detect of Metoprolol (neurotransmitter modulator) at 727 ng/L, the Marlborough West effluent sample had two detects, Sulfamethoxazole (antimicrobial) at 1096 ng/L and Gemfibrozil (lipid modifier) at 1030 ng/L and the Maynard effluent sample had three detects, Gemfibrozil (lipid modifier) at 1066ng/L, Valsartan (anti-hypertensive) at 1337 ng/L and Atenolol (neurotransmitter modulator) at 1663 ng/L.

## **Hormones**

The EPA analyzed the effluent samples for eight steroids and total NPs. Table 1.9 displays the eight steroid hormones consisting of estrogenic and androgenic compounds used in contraceptives as well as total NP concentrations. There were 13 detections and 23 concentrations below the method reporting limit (<MRL). Every effluent sample had at least two detections with Westborough and Hudson having the least (2) and Maynard having the most (6). Dihydrotestosterone (DHT) and progesterone (PROG) were not detected in any of the samples and NPs were detected in all of the samples. Detection concentrations across all compounds and effluents ranged from 1.58 ng/L (Androsterone, AND Marlborough West) to 21.4 (Estrone, E1 Maynard). EE2 was only detected in the Maynard effluent sample at a concentration of 2.68 ng/L, which has been shown to reduce fecundity in fish [53,54].

## **Discussion**

Results from the male hepatic VTG and CYP1A1 biological assays suggest that levels of estrogenic and dioxin like activity in the water samples collected from the Assabet River, its tributaries and WWTP effluents in 2010 and 2011 are very low. Both experiments conducted in 2010 and 2011 revealed no statistically significant increases in hepatic VTG expression in male Japanese Medaka using real-time RT-PCR and only one statistically significant difference in hepatic CYP1A1 expression was observed in 2011. The Hudson Instream sample collected in 2011 significantly induced CYP1A1 expression over control while no significant differences were seen in the 2010 experiment. The results from the chemical analyses performed by the USGS and USEPA were in agreement with our findings and overall few compounds were detected at low

concentrations. The results presented in this thesis suggest compounds in the Assabet River that bind the estrogen and aryl hydrocarbon receptors are not sufficiently concentrated or biologically active to induce statistically significant expression of VTG or CYP1A1. The results gathered from my experiments represent limited information about the biological impacts associated with the effluent dominated Assabet River. I will discuss the details of my findings as well as outline methodological improvements and future experiments that would give us a deeper understanding of threats to the Assabet River.

## **Biological Analyses**

### **Vitellogenin Expression in 2010 and 2011**

The hepatic VTG expression in male Medaka exposed to Assabet River and tributary water samples collected in 2010 revealed no statistically significant induction of VTG over control. The 1 nM E2 positive control induced VTG 22,000 fold above the laboratory water negative control. Notice that three of the water samples (A1 Impound Westboro, Sudbury Rd. Stow and Coles Brook) collected from the Assabet River have mean hepatic VTG expression levels below our laboratory water negative control. I interpret these results in two different ways. First, it is possible that certain characteristics of our laboratory water or rearing, including increased female to male ratios, causes slightly elevated levels of VTG in our male fish. Second, it may be the chemical mixtures of the four samples that caused a reduction in the expression levels. An *in vivo* study by Gräns and colleagues [55] demonstrated a 40% reduction in the expression of VTG in rainbow trout hepatocytes exposed to a combination of  $\beta$ NF and EE2. Neither the USGS nor the EPA analyzed the water samples for the compound  $\beta$ NF. The group with the

lowest mean expression level was the A1 Impound Westboro water sample, which was meant to serve as an upstream reference site. The reference site is intended to represent the least impacted location and I believe that is what our data suggest. The treatment group with the largest mean was RT 9 Westboro, which was intended to serve as a positive control because of its proximity to the Westborough WWTP. Although induction was not statistically significant this result matches our hypothesis that increased expression levels would be observed in this sample.

None of the water samples collected in 2011 revealed statistically significant induction of hepatic VTG induction in male Medaka. Notice that the expression levels of the 16.8 pM EE2 positive control were comparable to the laboratory water negative control, which was not the robust response we had anticipated. At the time of this experiment we were collaborating on a research project conducted by the EPA office of research and development (ORD). The EPA ORD is heavily involved in the EPA Endocrine Disruptor Screening Program (EDSP). This program is charged with providing information about their work screening chemicals for potential effects to the endocrine system. The EPA not only wants to provide information about which chemicals may be endocrine disruptors they also want to create protocols to test for endocrine disruptors. One protocol, the whole effluent toxicity (WET) test, exposes fathead minnow (*Pimephales promelas*) to whole effluent samples and measures mortality as well as VTG induction. In 2011 the ORD was optimizing this method with fathead minnow larvae and we contributed to their research efforts using effluents collected from the Assabet River WWTPs. The positive control for this acute WET test was 5 ng/L (16.8 pM), which is not only an environmentally relevant concentration of EE2 [4,56] but concentrations as low

as 1ng/L EE2 have been shown to cause effects in fathead minnow [57]. In accordance with the WET test EE2 concentration we used the same positive control believing it would elicit a robust response from our Japanese Medaka. We did not see a robust response with the 16.8 pM EE2 concentration and therefore included a 1nM E2 positive control from a different experiment in our analysis to demonstrate the response we customarily observe in our laboratory.

In general the means of the effluent water samples were higher than the means of the instream samples (except Hudson Instream). This is what one would expect to see when comparing the results of exposure to effluent and instream water samples because the instream samples would be subject to more dilution than samples taken from the effluent outfalls. The reference site, chosen a priori to represent a least impacted site, had one of the lowest expression means and the expression levels seen in our laboratory water negative control were comparable to all of the treatments. I believe this implies that hepatic VTG expression in male Medaka is low for all exposures but I will describe other possible reasons later in this discussion.

### **CYP1A1 Expression in 2010 and 2011**

Exposure of male Medaka to Assabet River and tributary water samples collected in 2010 did not result in statistically significant induction of CYP1A1 over control. Although I did not observe statistically significant differences in CYP1A1 expression levels across all groups the mean expression levels from four of the water samples were higher than the mean of the laboratory water negative control. Coles Brook is the only exposure where the mean expression level is below the negative control. This is in agreement with our rationale of selecting Coles Brook as a negative control site. Notice

the high degree of variability associated with the Fort Pond Brook group, which is due to the high expression of a single fish. If I could remove this sample from the analysis the mean of Fort Pond Brook would drop below the mean of the lab water but I cannot remove this individual because it is not a statistical outlier. Our results indicate very low dioxin like activity in the water samples collected in 2010, which is in agreement with the chemical analysis. The chemical analysis performed by the USGS revealed multiple compounds that bind the AhR such as the PAHs Benzo[a]pyrene, Fluoranthrene, Phenanthrene and Pyrene, but all were detected at very low concentrations. We know compounds that bind the aryl-hydrocarbon receptor were present in the water samples collected in 2010 however; the limited number of exposure experiments conducted did not reveal significant effects of CYP1A1 activating compounds.

The Hudson Instream water sample was the only exposure that revealed statistically significant CYP1A1 expression levels in 2011. In general the means of the effluent water samples (except Westboro Effluent) were higher than the means of the instream water samples (except Hudson Instream). This follows my predictions that the effluent samples would elicit a greater response than the instream samples. The mean of the reference site was among the lowest reinforcing its purpose of a least impacted site. The 1 mg/L  $\beta$ NF positive control for this exposure was not as robust as we had anticipated. This positive control was prepared based on information obtained from Cohen et al. [58] investigating CYP1A1 induction in Medaka. I believe this result is due to the natural variability, in physiology and sensitivity, of individual Medaka. High variability of VTG induction has been documented by Moffatt et al. [51] in a laboratory study using Medaka and by Vine et al. [59] in a wild fish population study of male Pike

*Escox lucius*). My results reveal a similar scenario with CYP1A1 induction in male Medaka. If I could to remove one male from the 1 mg/L  $\beta$ NF group expressing low levels of CYP1A1 and one male from the lab water group expressing high levels of CYP1A1 the 1 mg/L  $\beta$ NF group would have a mean expression higher than the lab water (0.001436 RFU and 0.0008803 RFU, respectively). I cannot, however remove either of these males because they are not statistical outliers. I believe these results indicate that my population size (N) for each group should have been larger. I chose to use five individuals in an effort to reduce the number of fish sacrificed while maintaining a large enough N to make statistical analyses. In future studies at least seven individuals should be used to ensure appropriate differences are revealed despite the natural variability among individuals [41].

## **Chemical Analyses**

### **USGS**

The USGS analyzed water samples collected from the five Assabet and tributary locations for organic wastewater contaminants (OWCs), dissolved organic carbon (DOC), nutrients, pharmaceuticals and hormones. OWCs are chemicals of varying sources such as agricultural, industrial and residential and varying uses such as insect repellents, plasticizers, phosphates, fire retardants, human and veterinary antibiotics and detergent metabolites [60]. The list of compounds analyzed by the USGS was specifically chosen because those compounds are routinely detected in the surface waters of North America [4]. OWCs and pharmaceuticals were detected in every sample and at all locations. More than half of the compounds (43) were not detected in any of the samples and of the thirty-nine compounds detected most were at very low concentrations. Possible

reasons for the low detection concentrations are degradation and dilution in WWTPs, septic systems or the aquifer. DEET is the main ingredient in most insect repellents, which are regularly used by humans and the likely reason for its high frequency of detection (82%). Bisphenol A, nonyl phenolic and octyl phenolic compounds, known endocrine disruptors [38,39], were detected with Bisphenol A having the highest frequency of detection at 64%. The highest Bisphenol A concentration was an estimated 0.360 µg/L, which is forty times lower than the concentration found to inhibit spermatogenesis in male fathead minnow [38]. Isophorone also had a frequency of detection of 64% with the highest concentration estimated at 0.016 µg/L, which is well below the 145 mg/L fathead minnow LC<sub>50</sub> observed by Cairns et al. in a 96-hour acute toxicity test [61]. Bromoform, a disinfection by product produced from adding chlorine to drinking water, was detected four times (36%) at two locations; it was detected in both samples from RT 9 Westboro and both samples from Sudbury Rd. The concentrations ranged from an estimated 0.019 µg/L to 22.3 µg/L, which was the highest concentration of any compound detected. The 22.3 µg/L concentration is below the 7 mg/L Atlantic Menhaden (*Brevoortia tyrannus*) LC<sub>50</sub> observed by Gibson et al. in a 96-hour acute toxicity test [62].

In order to determine the occurrence and distribution of EDCs in sections of the Assabet River Basin with different potential EDC sources the USGS analyzed the water samples for seventeen hormones or their metabolites. Four hormones were detected Estrone, Androstenedione, Androsterone and Testosterone and Androstenedione was detected at every location. Androstenedione is a metabolic intermediate in the production of testosterone, estrone and estradiol. The largest concentration of Androstenedione

detected was 1.45 ng/L, which is 276 times lower than the 401 ng/L observed by Stanko et al. [63] to masculinize female western mosquitofish, (*Gambusia affinis*). Androsterone was detected in two samples the largest of which (305 ng/L) is 1.4 times greater than the maximum concentration observed by Kolpin et al. [4] in a national reconnaissance study of pharmaceuticals, hormones and OWCS in U.S. streams susceptible to contamination. Overall the frequency of detection of the seventeen hormones from all locations was low (6.5%) as well as most of the concentrations detected. These results are in agreement with our biological analysis suggesting that the biological impacts to the Assabet River from the hormones analyzed are low.

#### **USEPA**

The EPA analyzed Assabet River WWTP effluents for twenty-nine pharmaceuticals (Table 1.8). For the discussion of these results I will refer to a paper published by Kolpin and colleagues [4] investigating 139 streams nation wide that are susceptible to contamination. They specifically chose a set of 95 compounds because

“... they are expected to enter the environment through common wastewater pathways, are used in significant quantities, may have human or environmental health implications, are representative or potential indicators of certain classes of compounds or sources, and/or can be accurately measured in environmental samples using available technologies.” [4]

Here I will discuss five of the compounds detected by the EPA and compare their findings to the maximum and median concentrations reported by Kolpin and colleagues. Sulfamethoxazole a veterinary and human antibiotic was detected in three of the Assabet River WWTP effluent samples with a maximum concentration of 1096 ng/L. This concentration is roughly half the maximum concentration (1900 ng/L) and seven times

greater than the median concentration (150 ng/L) reported by Kolpin and colleagues. Sulfamethoxazole is a veterinary and human antibiotic widely used therefore, it is not surprising to detect it in more than half of the EPA samples. Albuterol is an antiasthmatic prescription drug detected in two of the EPA samples with a maximum concentration of 20 ng/L. Albuterol was not detected in any of the 84 samples examined in the nationwide study, which is surprising considering the streams were specifically chosen for their susceptibility to contamination. Gemfibrozil a prescription lipid modifier was detected in three of the EPA samples two of which exceeded 1000 ng/L. The maximum concentration of Gemfibrozil identified in the EPA analysis was larger than both the 790 ng/L maximum concentration and the 48 ng/L median concentration found in the nationwide study. Acetaminophen is a nonprescription anti-inflammatory medication and the main compound in Tylenol. It had a 25% frequency of detection, which is comparable to 24% frequency of detection found in the national study. Ibuprofen an anti-inflammatory nonprescription drug found in medications such as Advil, Motrin and Midol was found in two of the EPA samples with a maximum concentration of 586 ng/L. The maximum and median concentrations from the national study are 1000 ng/L and 200 ng/L respectively. The maximum concentration detected by the EPA is half that of the national study and almost three times the median. The pharmaceuticals analyzed by the EPA are frequently detected in surface waters of the U.S. and represent a variety of prescription and nonprescription medications frequently consumed by humans.

The EPA also examined the four Assabet River WWTP effluents for 8 hormones (Table 1.9). Maynard had the most detections (5) and Westboro had the least (1). The largest concentration detected in the Maynard effluent was 21.4 ng/L of E1 (Estrone) and

the smallest was 2.34 ng/L of E2 (17  $\beta$ -Estradiol) both of which are endogenous reproductive hormones. Maynard also had the only detection of EE2, a synthetic estrogen used in contraceptives, with a concentration of 2.68 ng/L. A concentration of EE2 lower than 1 ng/L has been shown to reduce fecundity in fathead minnows [54]. The EPA detected few hormones at low concentrations in the Assabet River effluent samples.

### **Methodological Improvements and Future Directions**

The biological assays I used in my research were fairly simple yet highly informative. However, I do believe that methodological improvement could be made to enhance the results gained by this assay. In the following section I will highlight techniques that could be adjusted and describe future experiments that would improve our understanding of the biological impacts to the effluent-dominated Assabet River.

In my research I used an acute toxicity test exposing male Japanese Medaka to water samples for 72-hours with an 80% static renewal at 24 and 48 hours. Moffatt et al. [51] observed VTG induction in male medaka exposed to E2 for 24 hours in a laboratory exposure. They also observed a statistically significant decrease in VTG induction of fish that were exposed to E2 for 24 hours and then depurated (exposed to laboratory water) for 48 hours. The fish in my experiment were not depurated for any amount of time but I believe they may have experienced a similar effect due to the static renewal method (exchanging water at one specific time point) used and the relatively short half-lives of estrogenic compounds. The half-life of EE2 the most potent estrogen analyzed is approximately  $33 \pm 13$  hours [64]. Based on this data half of the EE2 in a given sample would lose its biological activity between 20 and 46 hours. The water samples used in the exposure experiments conducted in in 2010 and 2011 were collected over a period of

days and held at 4°C for the duration of the experiment. My experiments took place up to four days after the initial collection of water samples and renewals took place 24 and 48 hours after the experiment began. This means that some of my exposures were taking place six days after the initial collections. The hormone analysis by the USGS in 2010 was also performed days after collection and reported EE2 levels below the reporting limit for all samples. The low concentration of EE2 coupled with long holding times of the water samples may be a reason why we did not see hepatic VTG induction in male Medaka. The static renewal method used in my experiment is not representative of the exposure experienced by wild fish populations in the Assabet River. In the natural environment fish are continuously exposed to effluent constituents and the physical aspects of the ecosystem (photochemical reactions, aerobic and anaerobic conditions and sorption to solid particles and out of the water phase) contribute to the half-lives of estrogenic compounds [65-67]. In a static renewal exposure experiment fish are exposed to the chemicals in water in pulses; exposure ends when a compound is absorbed and if compounds are absorbed quickly exposure effectively ends until the next renewal [63]. A flow through exposure experiment in place of a static renewal experiment would more closely mimic exposure in the wild. Also, the fish in my experiments were adults exposed to Assabet River water samples for a short 72-hour period, an acute toxicity test. A chronic toxicity test with low-level exposures would be more realistic given the low levels of compounds detected from chemical analysis. Additionally a whole life cycle toxicity test, as opposed to using one life stage, would allow us to observe generational changes similar to what occurs in the wild [54]. The current research also cannot reveal effects of bioaccumulation from pharmaceuticals and hormones, which has been

demonstrated in a variety of aquatic organisms [68]. Future studies should consider these changes to the experimental design to more accurately represent the exposures experienced by fish and the biological impacts of these exposures in the Assabet River.

### **Summary**

The USGS and USEPA did a comprehensive analysis of pharmaceuticals, hormones and OWCs in samples collected from the Assabet River, its tributaries and treated wastewater effluent samples. The compounds analyzed are present in surface waters across the U.S. [4] and encompass several chemical classifications and sources. Overall 131 different compounds were analyzed by both agencies and 72 different compounds were detected. Of the 72 compounds detected the concentrations tended to be very low. The results of my biological assay investigating hepatic VTG and CYP1A1 gene expression levels in male Japanese Medaka revealed one statistically significant difference of CYP1A1 induction in fish exposed to the Hudson Instream sample.

This work suggests that estrogenic and CYP1A1 inducing compounds are present at very low biologically active concentrations in the Assabet River. This conclusion is based on my current results but I also believe there are several possible methodological improvements that would deepen our understanding of the potential biological impacts of pharmaceuticals, hormones and organic wastewater contaminants to the Assabet watershed.

**Table 1.1 Body weights of male Japanese Medaka used in the laboratory exposure experiment conducted in 2010.**

<b>2010</b>	<b>1nM E2</b>	<b>Laboratory Water</b>	<b>A1 Impound Westboro</b>	<b>Rt 9 Westboro</b>	<b>Sudbury Rd Stow</b>	<b>Coles Brook</b>	<b>Fort Pond Brook</b>
<b>Fish</b>	<b>Weight (g)</b>						
<b>1</b>	0.4054	0.3308	0.4332	0.2344	0.5524	0.4077	0.4588
<b>2</b>	0.3560	0.4809	0.5163	0.3319	0.4711	0.2966	0.2812
<b>3</b>	0.4749	0.4108	0.3304	0.4934	0.2530	0.4086	0.3476
<b>4</b>	0.4243	0.2860	0.3676	0.3894	0.3896	0.3077	0.3545
<b>5</b>	0.2816	0.2452	0.3096	0.2284	0.2647	0.2949	0.4180

**Table 1.2 Body weights of male Japanese Medaka used in the laboratory exposure experiment conducted in 2011.**

2011 Fish	Laboratory Water	16.8 pM EE2	Reference Site	Hudson Effluent	Marlboro Effluent	Westboro Effluent	Maynard Effluent	Hudson Instream	Marlboro Instream	Westboro Instream	Maynard Instream
1	0.3282	0.3400	0.2866	0.2509	0.5204	0.3038	0.3470	0.4033	0.2622	0.2717	0.2732
2	0.2856	0.2336	0.4506	0.4916	0.2907	0.3901	0.2357	0.4515	0.2845	0.2336	0.4267
3	0.3364	0.3136	0.2090	0.1934	0.3093	0.2810	0.3862	0.3109	0.2946	0.2418	0.4198
4	0.4878	0.2456	0.2986	0.2000	0.2019	0.3246	0.2500	0.2361	0.3537	0.2864	0.2210
5	0.3147	0.1917	0.2180	0.3246	0.3129	0.1600	0.2154	0.2434	0.1840	0.2839	0.3486

**Table 1.3 Quantity and quality of mRNA extracted from the livers of male Japanese Medaka used in exposure experiments conducted in 2010.**

<b>2010</b>	<b>Stock RNA Solution</b>		<b>Experimental RNA Solution</b>	
<b>Sample</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>
<b>1nM (272ng/L) E2</b>				
<b>1</b>	0.2875	1.96	0.1100	1.74
<b>2</b>	0.4247	1.97	0.0992	1.69
<b>3</b>	0.3330	1.94	0.1055	1.82
<b>4</b>	0.5154	1.72	0.1035	1.79
<b>5</b>	0.3676	1.98	0.0940	1.67
<b>Laboratory Water</b>				
<b>1</b>	0.0920	1.80	0.0920	1.80
<b>2</b>	0.2954	1.90	0.1123	1.67
<b>3</b>	0.4017	1.91	0.1133	1.63
<b>4</b>	0.2587	1.87	0.0830	1.76
<b>5</b>	0.1488	1.85	0.1074	1.66
<b>A1 Impound Westboro</b>				
<b>1</b>	0.3074	1.91	0.1108	1.90
<b>2</b>	0.3726	1.91	0.0977	1.78
<b>3</b>	0.3622	1.94	0.1269	1.92
<b>4</b>	No Sample			
<b>5</b>	0.2716	1.93	0.1144	1.91
<b>Rt 9 Westboro</b>				
<b>1</b>	0.1163	1.82	0.1163	1.82
<b>2</b>	0.1572	1.83	0.1136	1.87
<b>3</b>	0.3909	1.93	0.1200	1.88
<b>4</b>	0.4011	1.94	0.1079	1.90
<b>5</b>	0.1518	1.87	0.0999	1.93
<b>Sudbury Rd Stow</b>				
<b>1</b>	0.2383	1.90	0.1013	1.88
<b>2</b>	0.3369	1.94	0.1164	1.82
<b>3</b>	0.2026	1.94	0.1033	1.88
<b>4</b>	0.1394	1.94	0.0914	1.89
<b>5</b>	0.2113	1.92	0.0840	1.92
<b>Coles Brook</b>				
<b>1</b>	0.2020	1.78	0.0898	1.60
<b>2</b>	0.3268	1.92	0.0936	1.63
<b>3</b>	0.3590	1.89	0.0955	1.76
<b>4</b>	0.1732	1.78	0.0975	1.63
<b>5</b>	0.1570	1.79	0.1012	1.69
<b>Fort Pond Brook</b>				
<b>1</b>	0.2397	1.96	0.1267	1.87
<b>2</b>	0.1681	1.92	0.0970	1.86
<b>3</b>	0.1739	1.93	0.1073	1.87
<b>4</b>	0.3111	1.94	0.1173	1.94
<b>5</b>	0.1617	1.88	0.1113	1.88

**Table 1.4 Quantity and quality of mRNA extracted from the livers of male Japanese Medaka used in exposure experiments conducted in 2011.**

<b>2011</b>	<b>Stock RNA Solution</b>		<b>Experimental RNA Solution</b>	
<b>Sample</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>
<b>Laboratory Water</b>				
<b>1</b>	0.3485	1.94	0.0862	1.78
<b>2</b>	0.2227	1.91	0.1034	1.92
<b>3</b>	0.3744	1.94	0.1038	1.86
<b>4</b>	0.3523	1.92	0.1051	1.88
<b>5</b>	0.4633	1.93	0.1085	1.83
<b>16.8pM (5ng/L) EE2</b>				
<b>1</b>	0.2659	1.92	0.1034	1.93
<b>2</b>	0.3254	1.94	0.1074	1.90
<b>3</b>	0.4896	1.93	0.0908	1.86
<b>4</b>	0.3105	1.94	0.9267	1.75
<b>5</b>	0.3057	1.93	0.1096	1.86
<b>Reference Site</b>				
<b>1</b>	0.2305	1.89	0.0928	1.85
<b>2</b>	0.3422	1.87	0.1081	1.78
<b>3</b>	0.0776	1.89	0.0947	1.72
<b>4</b>	0.1090	1.85	0.1090	1.85
<b>5</b>	0.2747	1.87	0.1063	1.83
<b>Hudson Effluent</b>				
<b>1</b>	0.2600	1.88	0.0960	1.79
<b>2</b>	0.3396	1.90	0.0922	1.96
<b>3</b>	0.1233	1.84	0.1027	1.92
<b>4</b>	0.1562	1.88	0.1007	1.83
<b>5</b>	0.1520	1.90	0.0982	1.98
<b>Marlboro Effluent</b>				
<b>1</b>	0.2678	1.90	0.1125	1.93
<b>2</b>	0.1397	1.80	0.1037	1.95
<b>3</b>	0.1793	1.88	0.1090	1.96
<b>4</b>	0.1107	1.92	0.1107	1.92
<b>5</b>	0.2343	1.87	0.0967	1.81
<b>Westboro Effluent</b>				
<b>1</b>	0.0894	1.91		
<b>2</b>	0.1655	1.88	0.1037	1.97
<b>3</b>	0.1361	1.87	0.1036	1.80
<b>4</b>	0.1110	1.94	0.1110	1.94
<b>5</b>	0.1047	1.83	0.1047	1.83
<b>Maynard Effluent</b>				
<b>1</b>	0.2305	1.94	0.0858	1.46
<b>2</b>	0.0332	1.80	0.1061	1.74
<b>3</b>	0.2657	1.92	0.0959	1.87
<b>4</b>	0.2413	1.88	0.1132	1.82
<b>5</b>	0.1398	1.82	0.0909	1.88

**Table 1.4 Continued**

<b>2011</b>	<b>Stock RNA Solution</b>		<b>Experimental RNA Solution</b>	
<b>Sample</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>	<b>Conc (µg/µL)</b>	<b>260/280</b>
<b>Hudson Instream</b>				
<b>1</b>	0.1471	1.93	0.0952	1.84
<b>2</b>	0.2594	1.94	0.0884	1.96
<b>3</b>	0.1185	1.89	0.1044	1.83
<b>4</b>	0.1381	1.91	0.1086	1.99
<b>5</b>	0.2015	1.88	0.0935	1.85
<b>Marlboro Instream</b>				
<b>1</b>	0.1810	1.96	0.1094	1.83
<b>2</b>	0.1731	1.95	0.1001	1.89
<b>3</b>	0.1240	1.85	0.0899	1.93
<b>4</b>	0.2118	1.94	0.1078	1.93
<b>5</b>	0.0334	1.95	0.0979	1.84
<b>Westboro Instream</b>				
<b>1</b>	0.1667	1.83	0.0910	1.75
<b>2</b>	0.1422	1.86	0.0916	1.70
<b>3</b>	0.1606	1.82	0.0902	1.85
<b>4</b>	0.1823	1.84	0.0910	1.80
<b>5</b>	0.1096	1.77	0.1096	1.77
<b>Maynard Instream</b>				
<b>1</b>	0.0629	1.73	0.1080	1.79
<b>2</b>	0.4990	1.94	0.1015	1.91
<b>3</b>	0.3003	1.87	0.0947	1.85
<b>4</b>	0.1506	1.91	0.0905	1.93
<b>5</b>	0.5045	1.92	0.0992	1.80

**Table 1.5 Sequences of Japanese Medaka primers used for real time RT-PCR.**

<b>Accession #</b>	<b>Gene Name Species</b>	<b>Primer Sequences</b>	<b>Standard Curve RNA Unexposed</b>
AB074891	<b>Vitellogenin II</b> Medaka	F:gacagttcgtccgttcatc R:gagcaaaggaatggttcca	Female Liver
DQ118296	<b>Ribosomal Protein L7</b> Medaka	F:gagaaaaaggcccgttaaggt R:cctgatgacaaaggccagtt	Female Liver
AY297923	<b>CYP1A1</b> Medaka	F:ggcaagagtttggttcag R:attggccacagacacaaca	Female Liver

**Table 1.6 Nutrients, Organic Wastewater Contaminants (OWCs) and pharmaceuticals in Assabet River and tributary water samples analyzed by the USGS in 2010. The top two rows of each page are sample location and sample date Ref. [48].**

Analyte	AI Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook	
	8/3/10	8/9/10	8/3/10	8/6/10	8/4/10	8/11/10	8/4/10	8/9/10	8/2/10	8/5/10
<b>Nutrients</b>	mg/L									
Diss. Ammonia	E0.011	E0.010	0.062	0.047	E0.010	<0.02	0.073	0.078	0.027	0.032
diss nitrite	<0.002	<0.002	0.011	0.006	0.026	0.041	0.005	0.005	0.005	0.006
diss ammon + org N	0.459	0.475	1.050	0.866	0.600	0.652	0.650	0.743	0.251	0.266
tot ammon + org N	0.799	0.553	1.150	0.887	0.796	0.826	0.736	0.874	0.279	0.321
diss NO2 + NO3	<0.04	<0.04	26.100	27.500	4.220	7.920	0.087	0.064	1.940	1.740
total phosphorus	0.035	0.028	1.190	0.361	0.060	0.086	0.077	0.122	0.012	0.016
diss phosphorus	0.007	0.007	1.090	0.283	0.027	0.017	0.047	0.054	E0.004	0.007
ortho phosphorus	<0.008	<0.008	1.100	0.255	E0.005	E0.004	0.033	0.038	0.008	0.010
DOC	5.65	6.48	6.09	5.29	4.4	4.5	9.01	12	2.82	2.74
<b>OWCs and Pharmaceuticals</b>	µg/L									
Metalaxyl	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
2-Methylnaphthalene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Dichlorvos	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Bromacil	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
Bromoform	<0.2	<0.2	7.53	22.3	E0.028	E0.019	<0.2	<0.2	<0.2	<0.2
Anthracene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Benzo[a]pyrene	<0.2	<0.2	<0.2	<0.2	<0.2	E0.009	<0.2	<0.2	<0.2	<0.2
Diethyl phthalate	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Fluoranthene	<0.2	<0.2	<0.2	<0.2	<0.2	E0.019	E0.010	<0.2	E0.015	<0.2

RC, Replicate Concentration; <, less than reporting limit; E, Estimated

Table 1.6 Continued Ref. [48].

Analyte	A1 Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook	
	8/3/10	8/9/10	8/3/10	8/6/10	8/4/10	8/11/10	8/4/10	8/9/10	8/2/10	8/5/10
	µg/L									
Isophorone	E0.012	E0.011	E0.013	<0.2	E0.009	E0.016	E0.015	E0.013	<0.2	<0.2
Phenanthrene	<0.2	<0.2	<0.2	<0.2	<0.2	E0.008	<0.2	<0.2	<0.2	<0.2
Pyrene	<0.2	<0.2	<0.2	<0.2	<0.2	E0.014	E0.008	<0.2	E0.010	<0.2
Tetrachloroethylene	<0.4	<0.4	E0.007	E0.024	E0.010	E0.012	<0.4	<0.4	<0.4	<0.4
1,4-Dichlorobenzene	<0.2	<0.2	E0.074	E0.092	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Phenol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.555	<0.2	<0.2	<0.2
Naphthalene	<0.2	<0.2	<0.2	<0.2	E0.010	<0.2	<0.2	<0.2	<0.2	<0.2
Chlorpyrifos	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Pentachlorophenol	<1.60	<1.60	<1.60	<1.60	<1.60	<1.60	<1.60	<1.60	<1.60	<1.60
Prometon	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Carbaryl	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Atrazine	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Diazinon	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Triclosan	0.265	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Cotinine	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
2,6-Dimethylnaphthalene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
3-beta-Coprostanol	E1.66	<1.6	E1.53	E1.33	<1.6	<1.6	E2.51	<1.6	E1.41	E1.41
4-Cumylphenol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
4-n-Octylphenol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2

RC, Replicate Concentration; <, less than reporting limit; E, Estimated

Table 1.6 Continued Ref. [48].

Analyte	A1 Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook	
	8/3/10	8/9/10	8/3/10	8/6/10	8/4/10	8/11/10	8/4/10	8/9/10	8/2/10	8/5/10
	µg/L									
4-tert-Octylphenol	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
Acetophenone	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
AHTN	<0.2	<0.2	E0.112	E0.111	E0.004	<0.2	<0.2	<0.2	<0.2	<0.2
Anthroquinone	<0.2	<0.2	E0.042	E0.046	<0.2	E0.035	<0.2	<0.2	<0.2	E0.033
Benzophenone	<0.2	<0.2	E0.11	E0.11	E0.03	E0.03	<0.2	E0.007	<0.2	<0.2
beta-Sitosterol	E8.74	E0.369	E6.34	E4.61	<2.05	E0.650	E9.80	E0.550	E5.57	E5.93
Bisphenol A	E0.360	E0.019	E0.249	E0.215	<0.4	<0.4	E0.259	<0.4	E0.109	E0.129
Camphor	<0.2	E0.015	<0.2	<0.2	E0.016	E0.011	E0.019	E0.006	<0.2	<0.2
Cholesterol	E2.76	E0.36	E2.03	E1.82	E0.85	E0.38	E4.01	E0.39	E2.05	E1.83
d-Limonene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Indole	<0.2	E0.003	<0.2	<0.2	<0.2	E0.007	<0.2	E0.01	<0.2	<0.2
Isoborneol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Isoquinoline	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Menthol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Methyl salicylate	<0.2	<0.2	E0.018	<0.2	<0.2	<0.2	E0.016	<0.2	<0.2	<0.2
beta-Stigmastanol	E2.49	<1.7	E2.06	E1.26	E0.283	<1.7	E2.98	<1.7	E1.41	E1.93
Tributyl phosphate	<0.2	<0.2	E0.020	E0.016	<0.2	E0.024	<0.2	<0.2	<0.2	E0.011
Triphenyl phosphate	<0.2	<0.2	E0.03	E0.03	<0.2	E0.005	<0.2	<0.2	<0.2	<0.2
p-Cresol	<0.2	E0.011	E0.033	E0.045	<0.2	E0.014	<0.2	E0.012	<0.2	<0.2

RC, Replicate Concentration; <, less than reporting limit; E, Estimated

Table 1.6 Continued Ref. [48].

Analyte	A1 Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook	
	8/3/10	8/9/10	8/3/10	8/6/10	8/4/10	8/11/10	8/4/10	8/9/10	8/2/10	8/5/10
	µg/L									
Isopropylbenzene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Carbazole	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Caffeine	<0.2	E0.018	E0.022	<0.2	E0.014	E0.036	E0.045	<0.2	<0.2	<0.2
1-Methylnaphthalene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Metolachlor	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	E0.008	E0.004	<0.2	<0.2
5-Methyl-1H-benzotriazole	<1.6	<1.6	<1.6	E0.378	E0.196	E0.232	<1.6	<1.6	<1.6	<1.6
bis(2-Ethylhexyl) phthalate	<2	<5.03	E0.255	<2	<2	<2	<2	<2	E0.247	<2
Tris(2-butoxyethyl)phosphate	<0.2	<0.2	<0.2	<0.2	E0.422	E0.192	E0.212	<0.2	<0.2	<0.2
Tris(2-chloroethyl)phosphate	<0.2	<0.2	0.473	0.412	<0.2	E0.249	<0.2	<0.2	<0.2	<0.2
Triethyl citrate (ethyl citrate)	<0.2	<0.2	0.402	0.393	E0.018	<0.2	<0.2	<0.2	<0.2	<0.2
3,4-Dichlorophenyl isocyanate	<1.6	<1.6	E0.150	<1.6	<1.6	E0.024	<1.6	<1.6	<1.6	<1.6
3-Methyl-1(H)-indole (Skatole)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
3-tert-Butyl-4-hydroxy anisole (BHA)	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Tris(dichlorisopropyl)phosphate	<0.2	<0.2	0.508	0.471	E0.106	0.178	<0.2	<0.2	<0.2	E0.035
N,N-diethyl-meta-toluamide (DEET)	E0.113	0.446	<0.2	<0.2	E0.060	E0.066	E0.087	0.258	E0.032	E0.092
4-tert-Octylphenol monoethoxylate	E0.26	<1	<1	<1	<1	<1	<1	<1	<1	<1

RC, Replicate Concentration; <, less than reporting limit; E, Estimated

Table 1.6 Continued Ref. [48].

Analyte	A1 Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook		RC
	8/3/10	8/9/10	8/3/10	8/6/10	8/4/10	8/11/10	8/4/10	8/9/10	8/2/10	8/5/10	
	µg/L										
4-tert-Octylphenol diethoxylate	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Hexahydroxamethylcyclopent abenzopyran (HHCB)	<0.2	<0.2	0.748	0.729	E0.020	E0.021	<0.2	<0.2	<0.2	<0.2	<0.2
para-Nonylphenol (total) (branched)	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
4-Nonylphenol diethoxylate, (sum of all isomers)	E1.97	<3.2	<3.2	<3.2	<3.2	<3.2	E1.56	<3.2	<3.2	<3.2	<3.2
2,2',4,4'-Tetrabromo-diphenylether (PBDE 47)	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
4-Nonylphenol monoethoxylate, (sum of all isomers)	E0.63	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
Spiked Recoveries	%										
Bisphenol A-d3	94.94	65.44	76.71	78.75	60.45	67.76	87.68	63.62	81.45	84.19	55.84
Caffeine-C13	83.24	65.26	75.90	119.10	66.72	73.00	80.63	62.73	76.67	80.93	56.77
Decafluorobiphenyl	62.40	58.55	64.38	66.90	62.61	58.04	53.68	61.80	56.49	66.29	54.61
Fluoranthene-d10	86.21	68.58	81.54	78.42	73.80	67.44	76.61	62.94	83.70	85.77	58.85
Sample volume	982	968	969	939	969	944	917	970	981	948	938
Number of detects (not including nutrients)	11	9	23	19	16	25	16	9	9	7	5

RC, Replicate Concentration; <, less than reporting limit; E, Estimated

**Table 1.7 Hormones in Assabet River and tributary water samples analyzed by AXYS Analytical Services Ltd. in 2010. The first two rows are location and date Ref. [48].**

Hormone	AI Impound Westboro		RT 9 Westboro		Sudbury Rd. Stow		Fort Pond Brook		Coles Brook		BLANK
	8/4/14	8/10/14	8/4/14	8/7/14	8/5/14	8/12/14	8/5/14	8/10/14	8/3/14	8/6/14	
<b>17 <math>\alpha</math>-Dihydroequilin</b>	<0.401	<1.94	<1.16	<2.07	<0.700	<2.00	<0.667	<1.97	<0.402	<1.90	<0.674
<b>Equilenin</b>	<0.0802	<0.389	<0.133	<0.413	<0.140	<0.400	<0.133	<0.394	<0.0804	<0.380	<0.135
<b>Equilin</b>	<0.802	<3.89	<1.33	<4.13	<1.40	<4.00	<1.33	<3.94	<0.804	<3.80	<1.35
<b>17 <math>\beta</math>-Estradiol</b>	<0.401	<1.94	<0.664	<2.07	<0.700	<2.00	<0.667	<1.97	<0.402	<1.90	<0.674
<b>17 <math>\alpha</math>-Estradiol</b>	<0.401	<1.94	<0.664	<2.07	<0.700	<2.00	<0.667	<1.97	<0.402	<1.90	<0.674
<b>Estrone</b>	<0.401	<2.43	<0.664	<2.07	<0.700	<2.00	<b>0.853</b>	<1.97	<0.402	<1.90	<0.674
<b>17<math>\alpha</math>-Ethynylestradiol</b>	<0.501	<1.94	<0.830	<2.58	<0.876	<2.50	<0.834	<2.47	<0.503	<2.37	<.842
<b>Allyl Trenbolone</b>	<0.469	<0.370	<1.10	<0.319	<0.414	<0.311	<0.365	<0.504	<0.500	<0.284	<0.135
<b>Androstenedione</b>	<b>0.45</b>	<b>1.45</b>	<b>1.43</b>	<b>0.935</b>	<b>0.753</b>	<0.5389	<0.411	<b>0.76</b>	<0.384	<b>0.652</b>	<0.337
<b>Androsterone</b>	<2.01	<3.33	<3.32	<3.54	<3.50	<3.43	<3.34	<b>305</b>	<2.01	<b>194</b>	<3.37
<b>Desogestrel</b>	<12.0	ND	<19.9	ND	<21.0	ND	<20.0	ND	<12.1	ND	<20.2
<b>Estriol</b>	<1.60	<2.59	<2.66	<2.75	<2.80	<2.67	<2.67	<2.63	<1.61	<2.53	<2.69
<b>Mestranol</b>	<20.1	<15.5	<55.5	<19.1	<15.0	<17.1	<20.9	<19.5	<19.7	<18.0	<4.47
<b>Norethindrone</b>	<0.401	<0.648	<0.664	<0.688	<0.700	<0.667	<0.667	<0.657	<0.402	<0.633	<0.674
<b>Norgestrel</b>	<0.401	<0.648	<0.664	<0.688	<0.700	<0.667	<0.667	<0.657	<0.402	<0.633	<0.674
<b>Progesterone</b>	<0.0802	<0.130	<0.133	<0.138	<0.140	<0.133	<0.133	<0.131	<0.0804	<0.127	<0.135
<b>Testosterone</b>	<0.461	<0.498	<0.926	<b>0.686</b>	<0.371	<0.652	<0.509	<0.549	<0.497	<0.562	<0.135

ND, not detected; <, concentration below the reporting limit

**Table 1.8 Pharmaceuticals in effluent samples collected from four WWTPs on the Assabet River analyzed by the USEPA in 2010 Ref. [49].**

	Westboro	Marlboro West	Hudson	Maynard	Broad Mode of Action Class
Compound	ng/L				
Cimetidine	ND	22	ND	13	h2 anti-histamine
Ranitidine	ND	149	ND	120	h2 anti-histamine
Trimethoprim	93	183	18	175	Antimicrobial
Sulfamethoxazole	ND	1096	316	667	Antimicrobial
Atorvastatin	ND	21	ND	63	Lipid modifier
Gemfibrozil	ND	1030	20	1066	Lipid modifier
Theophylline	ND	ND	ND	19	Bronchodilator
Ibuprofen-2-hydroxy	ND	ND	ND	164	Inactive Metabolite
Acetaminophen	ND	ND	ND	129	Anti-inflammatory
Ibuprofen	ND	104	ND	586	Anti-inflammatory
Triamterene	32	27	132	180	Anti-hypertensive
Desmethyl-diltiazem	17	49	37	96	Anti-hypertensive
Diltiazem	60	175	39	297	Anti-hypertensive
Norverapamil	ND	ND	ND	18	Anti-hypertensive
Verapamil	51	25	9	61	Anti-hypertensive
Valsartan	ND	282	189	1337	Anti-hypertensive
Furosemide	ND	329	55	452	Anti-hypertensive
Metoprolol	727	505	553	612	NM
Propranolol	ND	32	12	46	NM
Carbamazepine	106	144	294	196	NM
Amitriptyline	ND	1	ND	ND	NM
Fluoxetine	ND	28	16	46	NM
Sertraline	ND	25	ND	ND	NM
Albuterol	ND	8	ND	20	NM
Atenolol	383	571	306	1663	NM
Oxycodone	98	75	33	181	NM
Amphetamine	ND	ND	ND	5	NM
Hydrocodone	31	ND	ND	ND	NM
10-hydroxy-amitriptyline	ND	2	ND	4	NM

ND, not detected above the reporting limit; NM, Neurotransmitter Modulator

**Table 1.9 Hormones and phenolic compounds in effluent samples collected from four WWTPs on the Assabet River analyzed by the USEPA in 2010 Ref. [49].**

	Westboro	Marlboro West	Hudson	Maynard
Hormone	ng/L			
<b>E1</b>	<MRL	8.83, S	<MRL,S	21.4
<b>E2</b>	<MRL	<MRL,S	<MRL, S	2.34
<b>EE2</b>	<MRL	<MRL,S	<MRL, S	2.68
<b>E3</b>	<MRL	<MRL, S	<MRL, S	5.5
<b>DHT</b>	<MRL	<MRL	<MRL	<MRL
<b>AND</b>	<MRL	1.58	<MRL	3.7
<b>TEST</b>	4.56	<MRL	20.3	<MRL
<b>PROG</b>	<MRL	<MRL	<MRL	<MRL
<b>Total NP</b>	5500	5700	5200	5900

<MRL, Less than method reporting limit; S, Surrogate recovery is out of criteria (60%–140%); E1, Estrone; E2, 17  $\beta$ -estradiol; EE2, 17  $\alpha$ -ethinylestradiol; E3, Estriol; DHT, Dihydrotestosterone; AND, Androsterone; TEST, Testosterone; PROG, Progesterone; NP, Nonylphenol.

**Table 1.10 General classifications and uses of analytes detected in Assabet River and tributary water samples analyzed by the USGS in 2010.**

<b>Compound Detected</b>	<b># of Detects</b>	<b>Class</b>	<b>Use</b>
<b>3-<math>\beta</math>-Coprostanol</b>	6	Fecal Steroid	Tracer for sewage
<b>AHTN/Tonalide</b>	4	Fragrance	Cosmetics
<b>Isophorone</b>	7	Industrial	Solvent
<b>Bisphenol A</b>	7	Industrial	Plasticizer
<b>Tributyl phosphate</b>	4	Industrial	Solvent, Plasticizer
<b>Triphenyl phosphate</b>	3	Industrial	Plasticizer
<b>Triethyl citrate (ethyl citrate)</b>	3	Industrial	Plasticizer, Food additive
<b>5-Methyl-1H-benzotriazole</b>	3	Industrial	Deicer
<b>Anthroquinone</b>	4	Industrial	Dyes
<b>Fluoranthene</b>	3	PAH <sup>1</sup>	Chemical By-Product
<b>Naphthalene</b>	1	PAH <sup>1</sup>	Chemical By-Product
<b>Phenanthrene</b>	1	PAH <sup>1</sup>	Chemical By-Product
<b>Pyrene</b>	3	PAH <sup>1</sup>	Chemical By-Product
<b>Benzo[a]pyrene</b>	1	PAH <sup>1</sup>	Chemical By-Product
<b>Metolachlor</b>	2	Pesticide	Herbicide
<b>Phenol</b>	1	Phenol	Synthetic Fibers
<b>Triclosan</b>	1	Phenol	Anti-Bacterial/Fungal
<b>p-Cresol</b>	5	Phenol	Disinfectant, Dodorizer
<b>Tris(2-butoxyethyl)phosphate</b>	3	Phosphate	Fire Retardant
<b>Tris(2-chloroethyl)phosphate</b>	3	Phosphate	Fire Retardant
<b>Tris(dichlorisopropyl)phosphate</b>	6	Phosphate	Fire Retardant
<b>Bis(2-Ethylhexyl)phthalate</b>	2	Phthalate	Plasticizer
<b>Cholesterol</b>	11	Plant/Animal Steroid	Unknown
<b><math>\beta</math>-Sitosterol</b>	9	Plant Steroid	Pharmaceuticals
<b><math>\beta</math>-Stigmastanol</b>	7	Plant Steroid	Pharmaceuticals

<sup>1</sup>Polycyclic Aromatic Hydrocarbon

**Table 1.10 Continued**

<b>Compound Detected</b>	<b># of Detects</b>	<b>Class</b>	<b>Use</b>
<b>DEET</b>	9	PPCPs <sup>2</sup>	Insect Repellent
<b>HHCB</b>	4	PPCPs <sup>2</sup>	Fragrance
<b>Methyl salicylate</b>	2	PPCPs <sup>2</sup>	Fragrance
<b>4-tert-Octylphenol monoethoxylate</b>	1	PPCPs <sup>2</sup>	Detergent Metabolite
<b>4-Nonylphenol diethoxylate,</b>	2	PPCPs <sup>2</sup>	Detergent Metabolite
<b>4-Nonylphenol monoethoxylate</b>	1	PPCPs <sup>2</sup>	Detergent Metabolite
<b>Benzophenone</b>	5	PPCPs <sup>2</sup>	Sunscreen
<b>Caffeine</b>	5	PPCPs <sup>2</sup>	Stimulant
<b>Indole</b>	3	PPCPs <sup>2</sup>	Fragrance, Drugs
<b>Camphor</b>	5	Terpenoid	Moth Repellent
<b>3,4-Dichlorophenyl isocyanate</b>	2	Unknown	Unknown
<b>Bromoform</b>	4	VOC <sup>3</sup>	Chemical By-Product
<b>Tetrachloroethylene</b>	4	VOC <sup>3</sup>	Chemical By-Product
<b>1,4-Dichlorobenzene</b>	2	VOC <sup>3</sup>	Chemical By-Product
<b>Total</b>	<b>149</b>	<b>12</b>	<b>~21</b>

<sup>2</sup>Pharmaceuticals and Personal Care Products; <sup>3</sup>Volatile Organic Carbons

**Table 1.11** Pollutants analyzed in Assabet River samples by the USGS in 2010 listed on the ATSDR 2011 Substance Priority List (Column 1), the EPA 126 Priority Pollutants List (Column 2) and pollutants that are on both lists (Column 3).

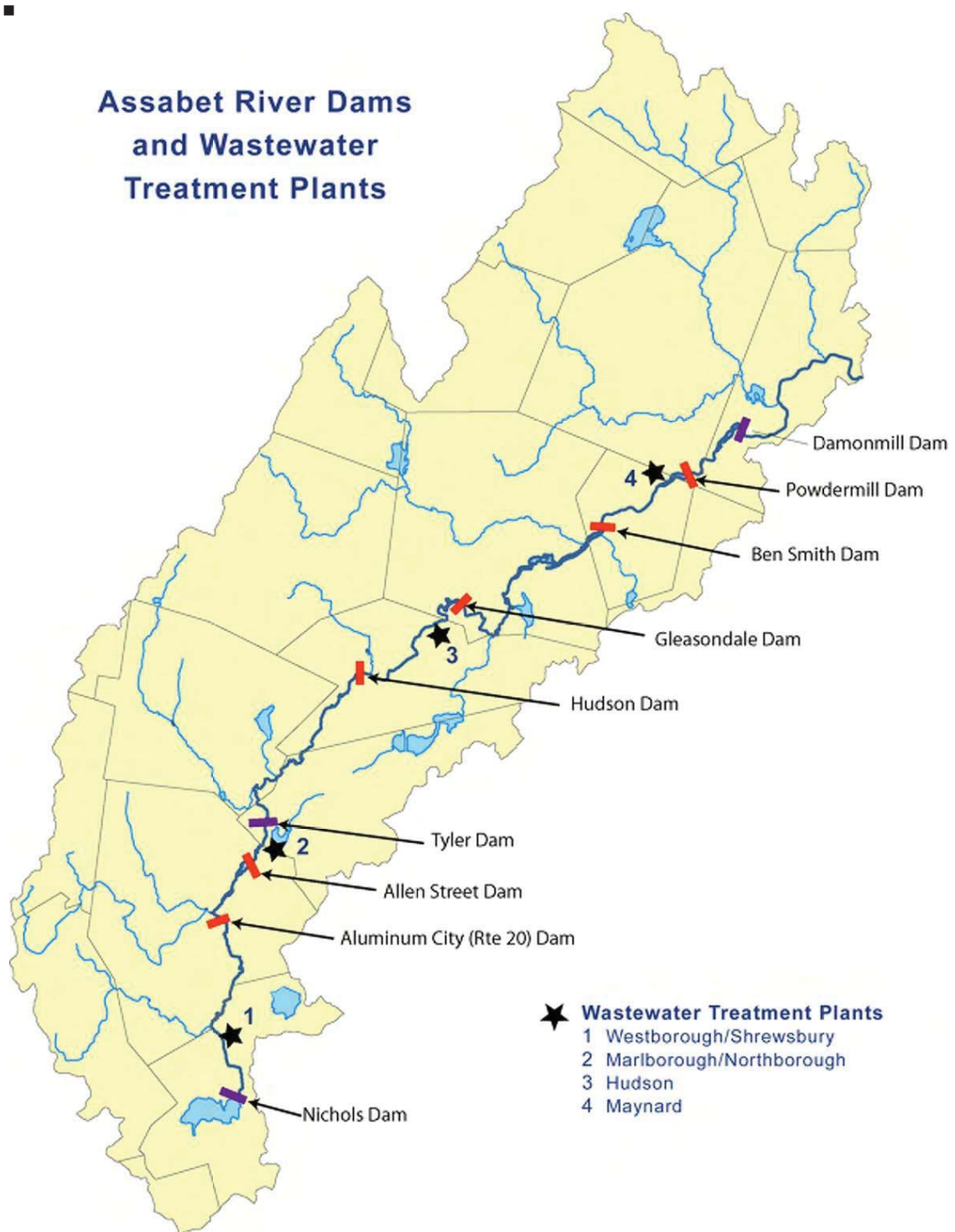
The ATSDR 2011 Substance Priority List		EPA 126 Priority Pollutants		Compounds on Both Lists	
#	Name	#	Name		Name
8	Benzo(A)Pyrene <sup>1</sup>	27	1,4-Dichlorobenzene <sup>2</sup>	1	Benzo(A)Pyrene <sup>1</sup>
33	Tetrachloroethylene (PERC) <sup>2</sup>	39	Fluoranthene <sup>1</sup>	2	Tetrachloroethylene (PERC) <sup>2</sup>
41	Diazinon <sup>3</sup>	47	Bromoform (Tribromomethane) <sup>2</sup>	3	Pentachlorophenol <sup>3,4,7</sup>
53	Pentachlorophenol <sup>3,4,7</sup>	54	Isophorone <sup>4</sup>	4	Bis(2-ethylhexyl) Phthalate <sup>5</sup>
65	Chlorpyrifos <sup>3</sup>	55	Naphthalene <sup>6</sup>	5	Naphthalene <sup>6</sup>
76	Di(2-Ethylhexyl)Phthalate (DEHP) <sup>5</sup>	64	Pentachlorophenol <sup>3,4,7</sup>	6	Fluoranthene <sup>1</sup>
80	Naphthalene <sup>6</sup>	65	Phenol <sup>7</sup>	7	1,4-Dichlorobenzene <sup>2</sup>
133	Fluoranthene <sup>1</sup>	66	Bis(2-ethylhexyl) Phthalate <sup>5</sup>	8	Phenol <sup>7</sup>
158	2-Methylnaphthalene <sup>6</sup>	70	Diethyl Phthalate <sup>3,4,5</sup>	9	Bromoform <sup>2</sup>
159	1,4-Dichlorobenzene <sup>2</sup>	73	Benzo(a)Pyrene (3,4-benzo-pyrene) <sup>1</sup>	10	Phenanthrene <sup>1</sup>
179	Phenol <sup>7</sup>	78	Anthracene <sup>1</sup>	11	Pyrene <sup>1</sup>
193	Dichlorvos <sup>3</sup>	81	Phenanthrene <sup>1</sup>	<b>11</b>	<b>Total Compounds</b>
202	Bromoform <sup>2</sup>	84	Pyrene <sup>1</sup>		
246	Phenanthrene <sup>1</sup>	85	Tetrachloroethylene (PERC) <sup>2</sup>		
253	Pyrene <sup>1</sup>	<b>14</b>	<b>Total Compounds</b>		
266	Carbazole <sup>8</sup>				
267	Metolachlor <sup>3</sup>				
270	Carbaryl <sup>3</sup>				
<b>18</b>	<b>Total Compounds</b>				

<sup>1</sup>PAH, <sup>2</sup>VOC, <sup>3</sup>Pesticides, <sup>4</sup>Industry/Commercial, <sup>5</sup>Phthalates, <sup>6</sup>Hydrocarbons, <sup>7</sup>Phenols/Phenoxy Acids, <sup>8</sup>Heterocyclic Aromatic Organic Compound

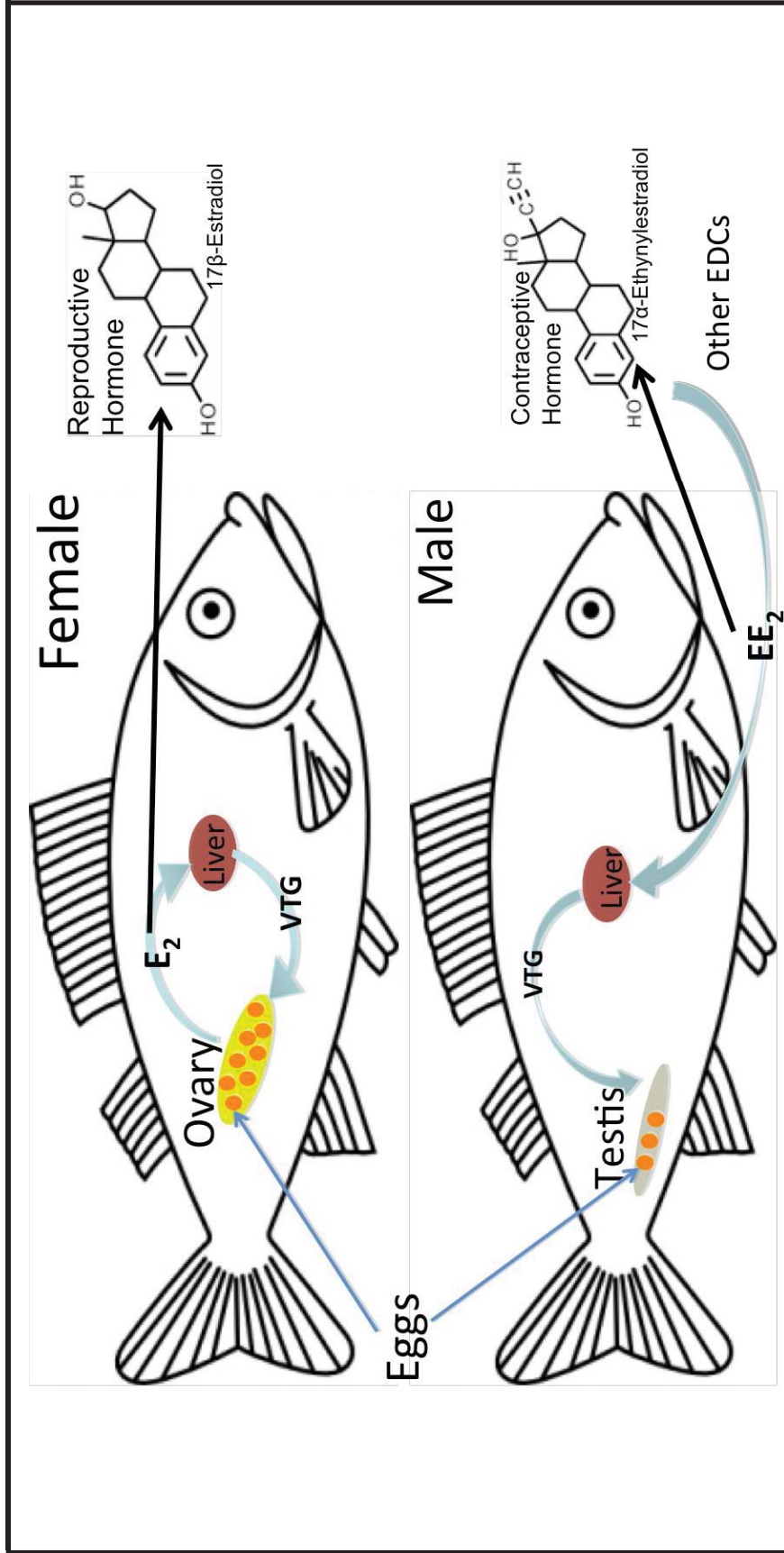
**Table 1.12 Sources and uses of the hormones analyzed by AXYS Analytical Services Ltd. in 2010.**

<b>17 <math>\alpha</math>-Dihydro-equilin</b>	Conjugated equine estrogen <sup>1,A</sup>
<b>Equilenin</b>	Weak estrogen from urine of pregnant mares <sup>1,A</sup>
<b>Equilin</b>	Equine estrogen (Used for human hormone replacement therapy) <sup>1,A</sup>
<b>Estrone</b>	E1, endogenous estrogen <sup>2,A</sup>
<b>17 <math>\beta</math>-Estradiol</b>	E2, Estradiol, natural estrogen in humans mainly females, metabolic product of testosterone <sup>2,A</sup>
<b>Estriol</b>	E3, one of 3 main estrogens produced by the body <sup>2,A</sup>
<b>Testosterone</b>	Natural androgen <sup>2,B</sup>
<b>Androsterone</b>	Weak endogenous androgen <sup>2,B</sup>
<b>Progesterone</b>	Progestogen; steroid hormone involved in female menstrual cycle, pregnancy and embryogenesis; major naturally occurring human progestogen <sup>2,D</sup>
<b>17 <math>\alpha</math>-Estradiol</b>	Medication, antiandrogenic, used to treat hair loss <sup>3,C</sup>
<b>17<math>\alpha</math>-Ethinylestradiol</b>	EE2, synthetic estrogen used in oral contraceptives <sup>4,A</sup>
<b>Mestranol</b>	Synthetic estrogen used in the first oral contraceptives <sup>4,A</sup>
<b>Allyl Trenbolone</b>	Progestin; synthetic progestogen, progestogens are one of 5 major classes of steroid hormones <sup>4,D</sup>
<b>Desogestrel</b>	Progestin; synthetic progestogen; third generation oral contraceptive <sup>4,D</sup>
<b>Norethindrone</b>	Progestin; synthetic progestogen, used in oral contraceptives <sup>4,D</sup>
<b>Norgestrel</b>	Progestin; synthetic progestogen, used in hormonal contraceptives <sup>4,D</sup>
<b>Androstenedione</b>	Intermediate for the production of testosterone, estrone and estradiol*

<sup>1</sup>Equine; <sup>2</sup>Natural/Endogenous; <sup>3</sup>Medication; <sup>4</sup>Synthetic; <sup>A</sup>Estrogen; <sup>B</sup>Androgen; <sup>C</sup>Antiandrogen; <sup>D</sup>Progestogen; \*, Other

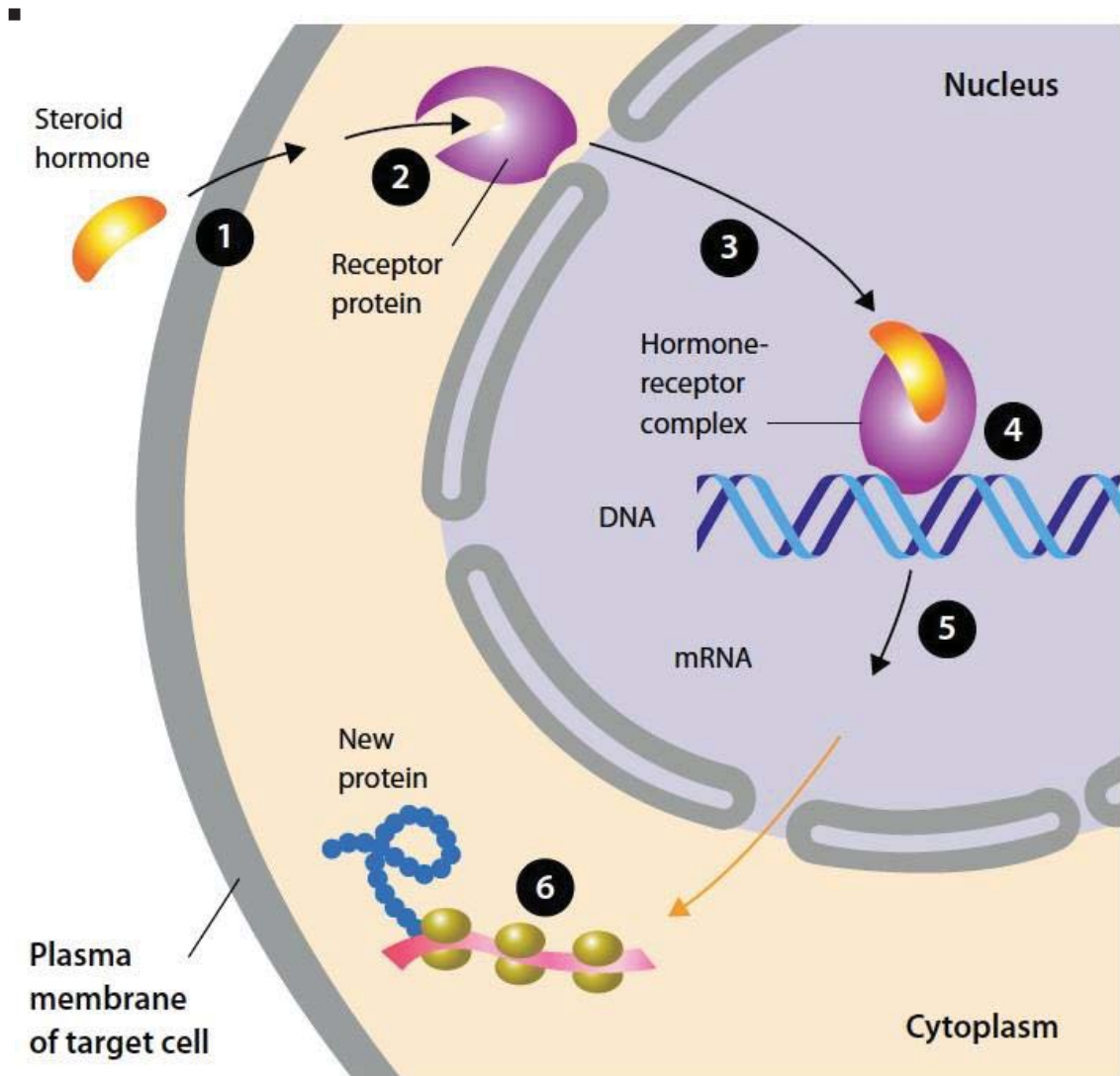


**Figure 1.1** Map of dams and wastewater treatment plants on the Assabet River. Red rectangles are mill dams and the purple rectangle, Damonmill Dam, is also a mill dam. The four major wastewater treatment plants are indicated by a black star. Map courtesy of OARS.



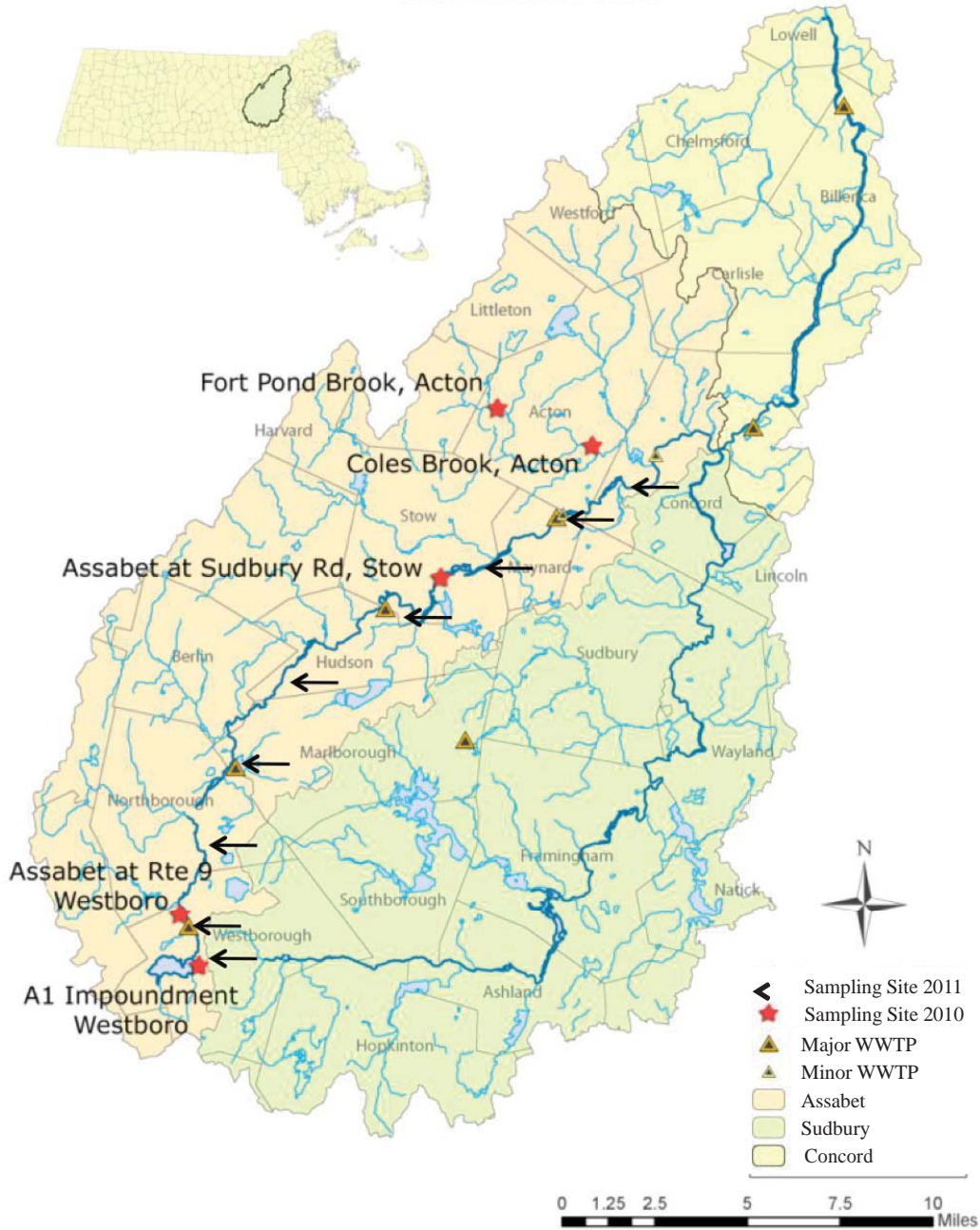
**Figure 1.2 Schematic representing the pathway of endogenous and exogenous estrogens in male and female Japanese**

**Medaka.** When female medaka are ready to reproduce their ovaries produce the natural estrogen E<sub>2</sub> (17β-Estradiol). E<sub>2</sub> travels to the liver where it stimulates vitellogenin production. The VTG protein travels to the ovaries where it stimulates egg production. When males are exposed to exogenous estrogens such as the synthetic estrogen EE<sub>2</sub> (17α-Ethynylestradiol) a similar pathway is activated. EE<sub>2</sub> travels to the liver where it stimulates VTG production that we measure using real time RT-PCR. VTG, in extreme cases, causes egg production in the testes of male fish (ovotestes).



**Figure 1.3 Schematic of protein activation pathway by steroid hormones.** This steroid hormone (1) represents natural and synthetic estrogens as well as estrogen mimics. The hormone (1) binds the receptor (2) shown on the cytoplasmic side of the nucleus. The hormone-receptor complex (3) dimerizes (not shown) and binds the estrogen response element on the DNA. This binding activates the gene (4, in this case vitellogenin, VTG). The gene transcribes mRNA (5, which we isolate from the livers of medaka). Translation of the mRNA produces the VTG protein (6), which can travel to the ovaries or testes to stimulate egg production [Ref. 8].

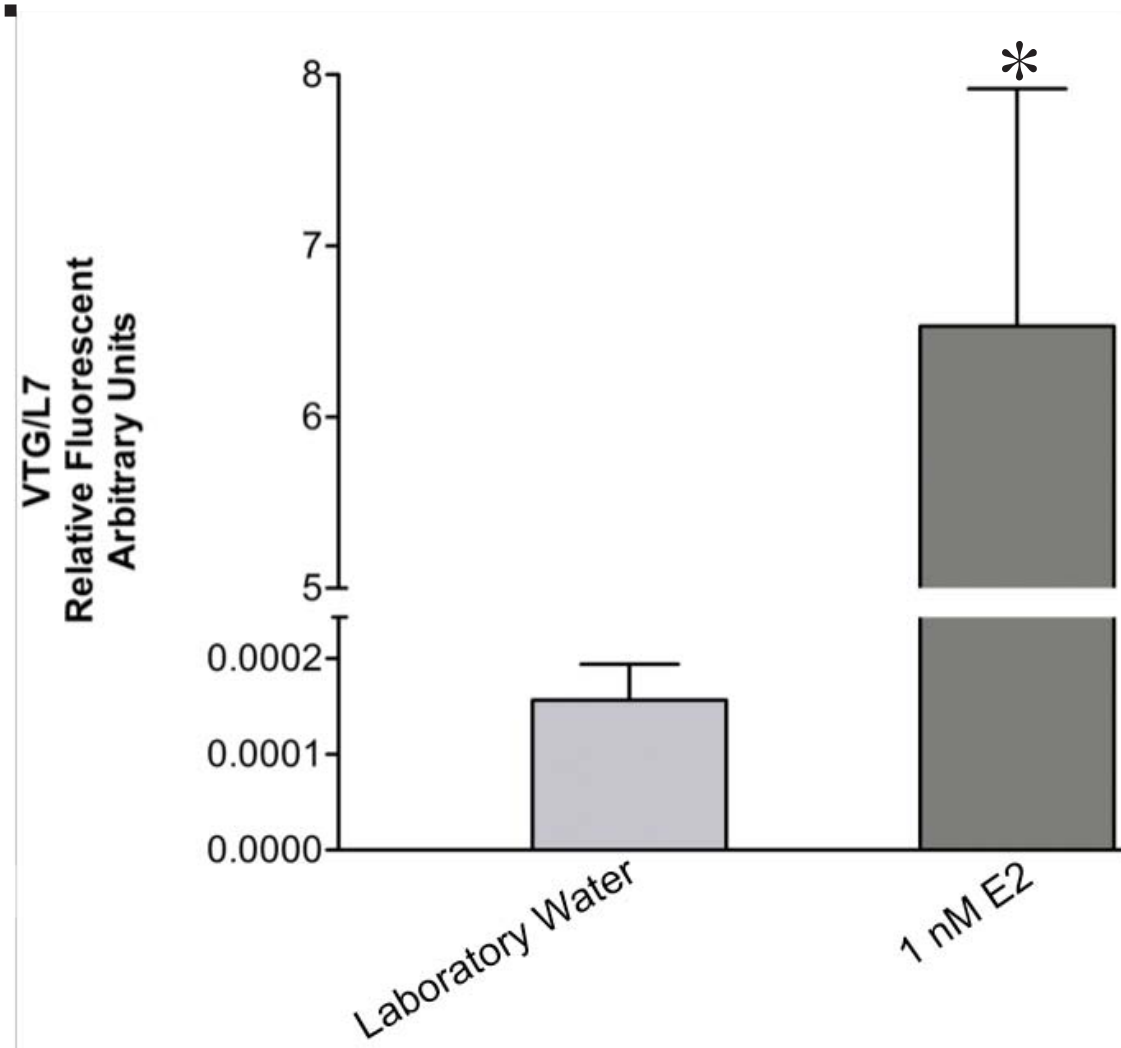
## Sudbury, Assabet and Concord Watershed Massachusetts



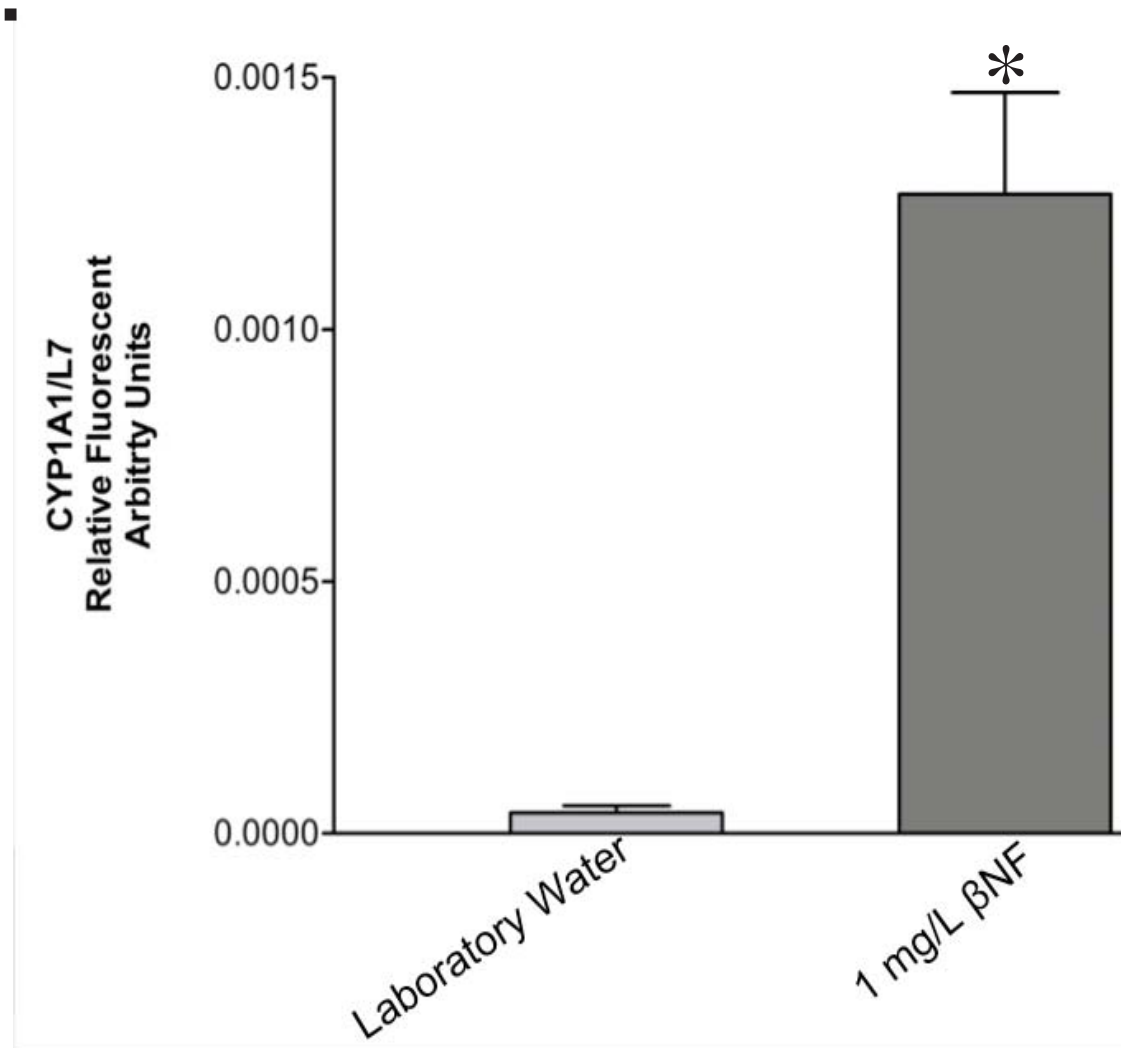
**Figure 1.4** Map of sampling sites along the Assabet River from 2010 and 2011. Map of the Assabet, Sudbury and Concord river watersheds. Red stars denote sampling sites from 2010, black arrows denote sampling sites from 2011 and triangles denote wastewater treatment plants.



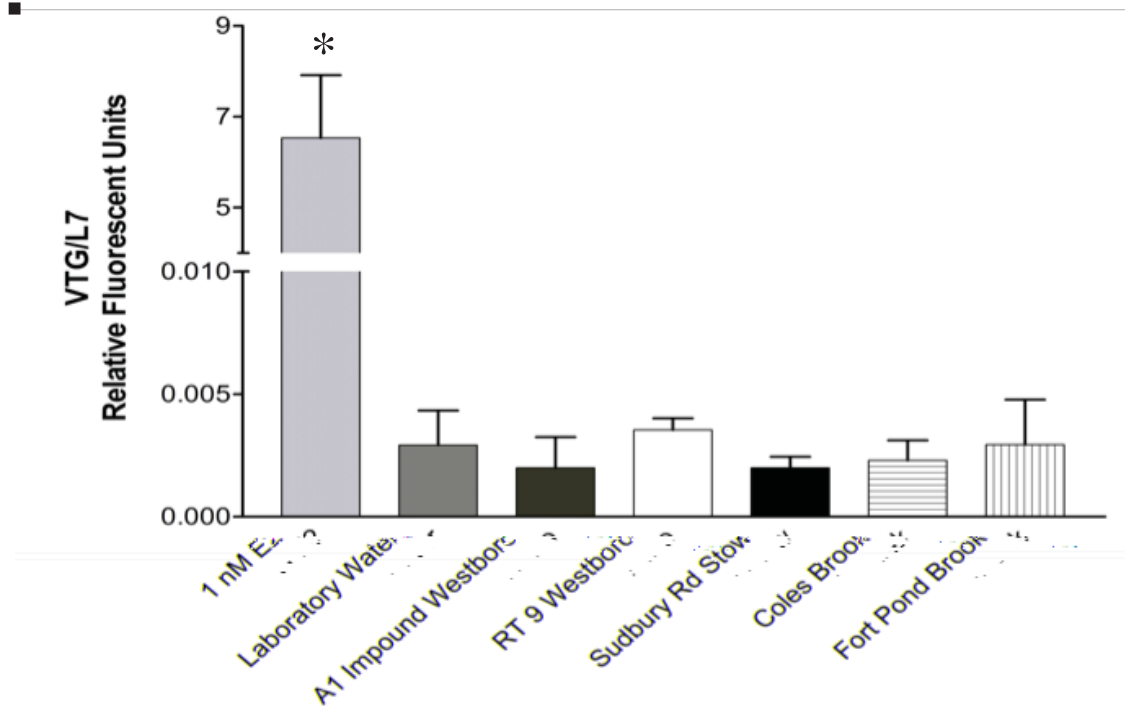
**Figure 1.5 Experimental set up of the exposures conducted in 2011.** Medaka were exposed to eleven different water samples: 1 positive and 1 negative control; 1 reference sample; 4 effluent samples; 4 instream samples. Water clarity varied among the eleven water samples. Photograph courtesy of Eva Browne.



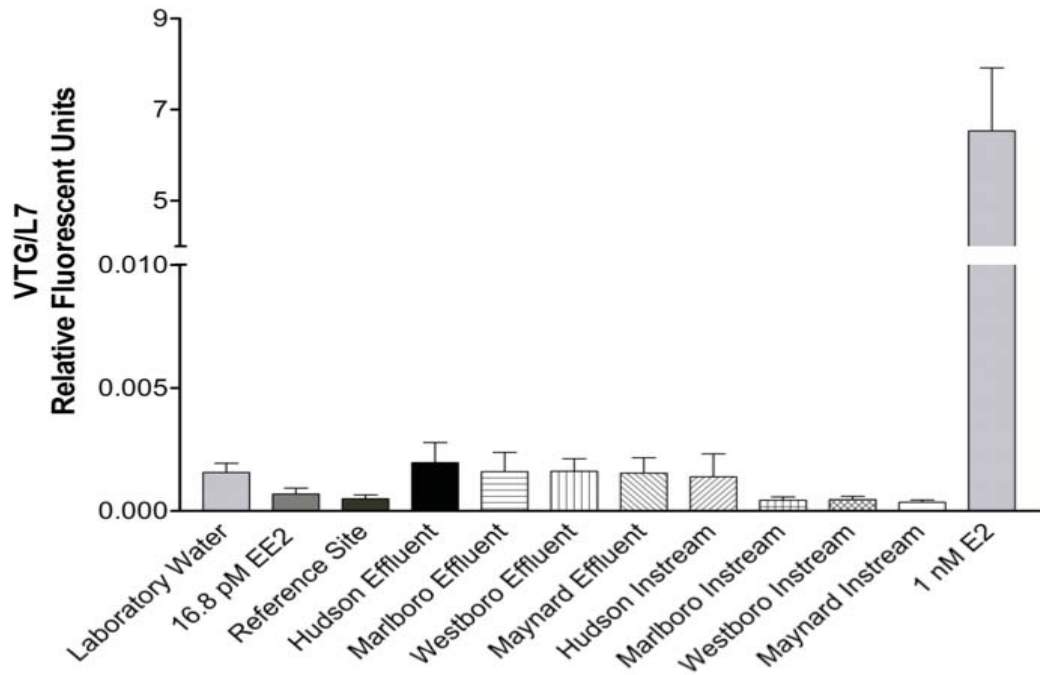
**Figure 1.6** Expression of hepatic Vitellogenin (VTG) in male Medaka exposed to a 1 nM E2 positive control. Male medaka (n = 5 /group) were exposed to a Laboratory Water negative control and a 1 nM (272 ng/L) E2 positive control. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, fish were sacrificed and expression of VTG in their livers was measured using real time RT-PCR. Relative VTG levels are shown normalized to the housekeeping gene L7. An asterik denotes statistical significance.



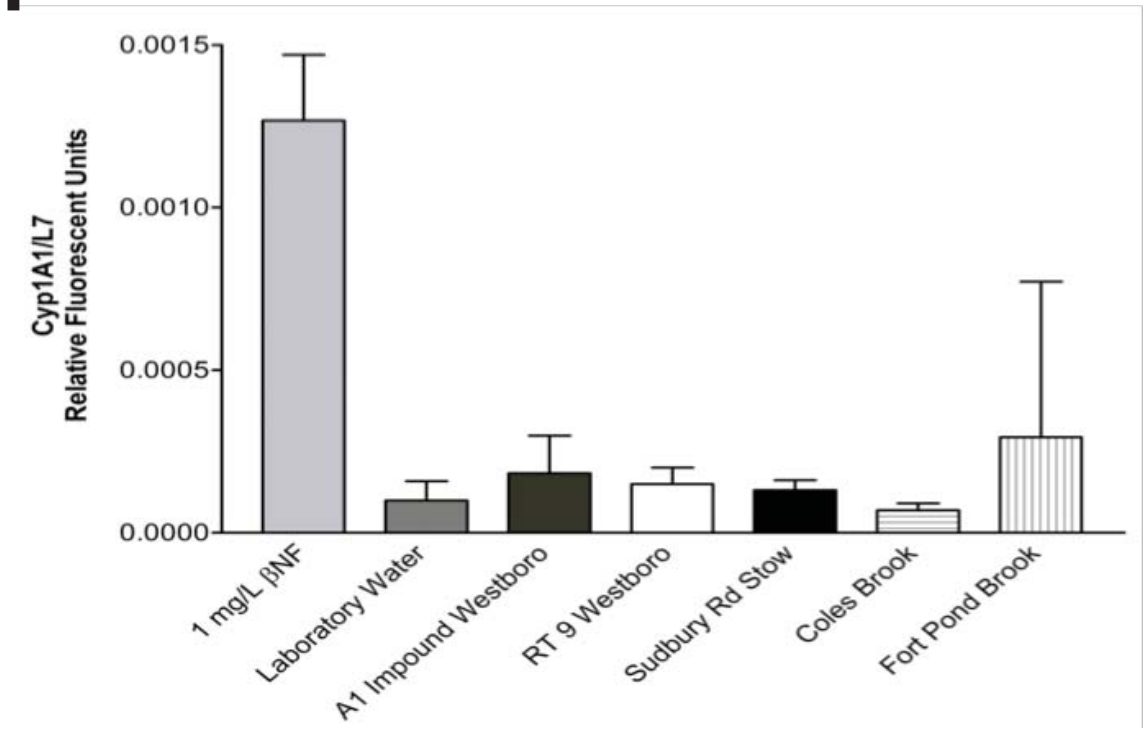
**Figure 1.7** Expression of hepatic Cytochrome P450-1A1 (CYP1A1) in male Medaka exposed to a 1 mg/L βNF positive control. Male medaka (n = 5 /group) were exposed to a Laboratory Water negative control and a 1 mg/L *beta*-Naphthoflavone (βNF) positive control. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, fish were sacrificed and expression of CYP1A1 in their livers was measured using real time RT-PCR. Relative CYP1A1 levels are shown normalized to the housekeeping gene L7. An asterik denotes statistical significance.



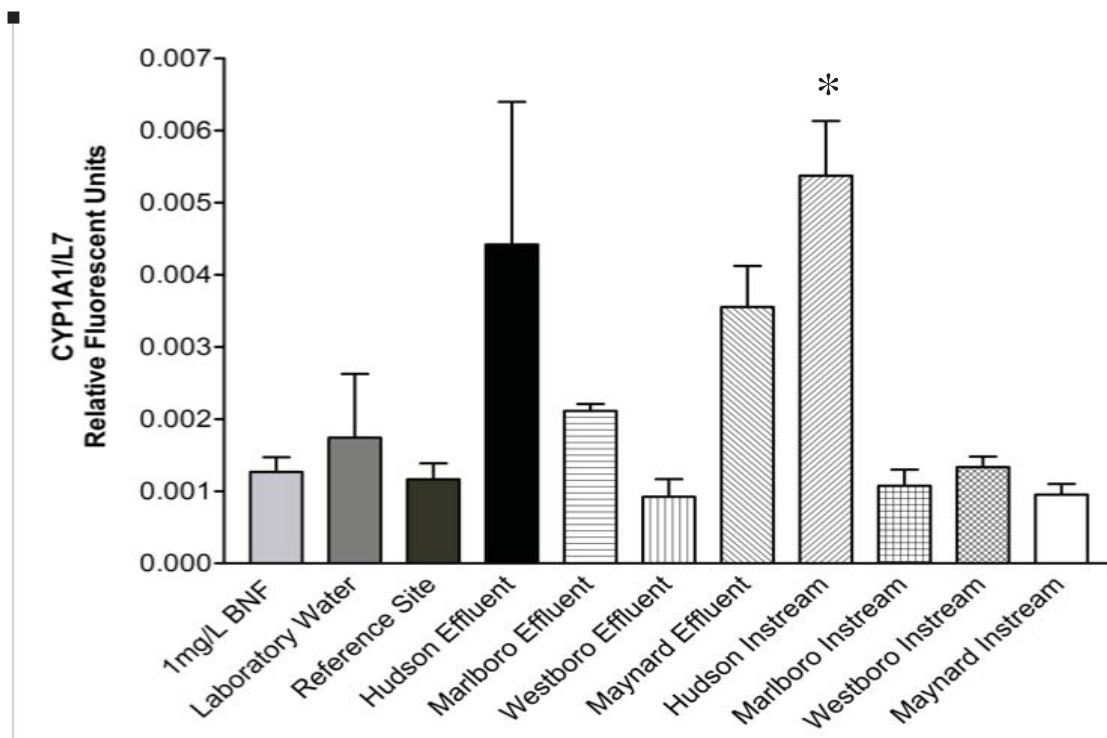
**Figure 1.8 Estrogenic activity of water samples collected from the Assabet River in the summer of 2010.** Male medaka (n = 5 /group, except A1 where n = 4) were exposed to either a positive (1nM or 272 ng/L E2) control, a negative (Laboratory Water) control or water collected from one of five locations along the Assabet River. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, fish were sacrificed and expression of vitellogenin (VTG) in their livers was measured using real time RT-PCR. Relative VTG levels are shown normalized to the housekeeping gene L7.



**Figure 1.9** Estrogenic activity of water samples collected from the Assabet River in the summer of 2011. Male medaka ( $n = 5$  /group) were exposed to either a negative (Laboratory Water) control, a positive (16.8 pM or 5 ng/L EE2) control, a reference site, effluents from one of four waste water treatment plants discharging into the Assabet River, or water collected from one of four locations along the Assabet River. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, fish were sacrificed and expression of vitellogenin (VTG) in their livers was measured using real time RT-PCR. Relative VTG levels are shown normalized to the housekeeping gene L7. Data from a 2010 1 nM (272 ng/L) E2 positive control are shown for comparison.



**Figure 1.10** Cytochrome P450-inducing activity of water samples collected from the Assabet River in the summer of 2010. Male medaka (n = 5 /group, except A1 where n = 4) were exposed to either a negative (Laboratory Water) control, or water collected from one of five locations along the Assabet River. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, the fish were sacrificed and expression of Cytochrome P450-1A1 (Cyp1A1) in livers was measured using real time RT-PCR. Relative Cyp1A1 levels are shown normalized to the housekeeping gene L7. A positive (1mg/L *beta*-Naphthoflavone,  $\beta$ NF) control conducted in November 2011 is shown for comparison.



**Figure 1.11** Cytochrome P450-inducing activity of water samples collected from the Assabet River in the summer of 2011. Male medaka (n = 5 /group) were exposed to either a negative (Laboratory Water) control, a reference site, effluents from one of four wastewater treatment plants discharging into the Assabet River, or water collected from one of four locations along the Assabet River. After 72-hour static exposures with 80% water renewals at 24 and 48 hours, fish were sacrificed and expression of Cytochrome P450-1A1 (CYP1A1) in their livers was measured using real time RT-PCR. Relative CYP1A1 levels are shown normalized to the housekeeping gene L7. A positive (1mg/L *beta*-Naphthoflavone,  $\beta$ NF) control conducted in November 2011 is shown for comparison. An asterik denotes statistical significance.

## CHAPTER 2

### INVESTIGATING THE EFFECTS OF DIFFERENT LAND USE/LAND COVER PROFILES ON WATER QUALITY IN THE ASSABET RIVER USING GIS

#### **Introduction and Background**

Geographic Information Systems (GIS) is an analytical tool used throughout several fields of research including environmental science and aquatic toxicology. GIS has many capabilities such as locating landmarks, determining population and identifying land use/land cover (LU/LC). The databases available for North America are numerous and detailed. The accuracy of locations and areas are within inches depending on when the database were last updated. The USGS and USEPA along with several other agencies, government and other, are compiling and updating information daily. There are many types of analyses that are possible using GIS including how LU/LC and population might affect water quality and how it relates to human and environmental health [69]. I compiled the data used for the present analyses in the spring of 2012 using the most up-to-date information available at that time. The combination and manipulation of GIS tools and data presented here represent an in-depth analysis of population and LU/LC relating to water quality on the Assabet River.

#### **Materials and Methods**

The information contained in GIS databases comes in the form of data layers and all of the data layers used for the following analyses were downloaded from [www.mass.gov/mgis](http://www.mass.gov/mgis), a Massachusetts state government database. The data layers used in the present study include water body information, population statistics (obtained from the most recent U.S. census), town locations and sizes as well as land use cover. I assembled

water body data to create one Assabet River layer including all its lakes and ponds. Next, I gathered population statistics of the nine towns that border the river, using the censuses conducted in 1980, 1990, 2000 and 2010 covering a period of thirty years. Finally, I downloaded land use cover data. The land use cover data layer contains all parcels of land for a given area along with the major use for each parcel; e.g. recreational or commercial. Water body, population and LU/LC data as well as numerous GIS tools were utilized to perform an intensive analysis relating to water quality on the Assabet River. The results from the analyses indicate potential impacts on the water quality of the Assabet River.

## **Results**

### **Population**

The total population of all nine towns along the mainstem of the Assabet River increased in the thirty years between 1980 and 2010 (Table 2.1 and Figure 2.1). Marlborough had the largest increase in total population from 31,550 people in 1980 to 38,499 people in 2010 for a total population increase of 6,949 people. Maynard had the smallest population increase from 9,822 in 1980 to 10,106 in 2010 for a total population increase of 284. The increase in total population for all nine towns is 24,905 increasing from 124,238 in 1980 to 149,143 in 2010. An increase of more than 20,000 residents has increased the total volume of wastewater that WWTPs receive. This increase strains WWTPs, and along with other strains, such as stricter EPA regulations e.g. the decrease in seasonal phosphorus concentrations allowed in treated effluent, forces plants to increase their size, upgrade their technology or both. The four major WWTPs along the Assabet River are Westborough, Marlborough, Hudson and Maynard, all of which have

National Pollutant Discharge Elimination System (NPDES) permits with standards that require them to upgrade their facilities. Population increases are a major strain on WWTPs, the Assabet River and its watershed but land use cover is the variable that will be extensively analyzed in the following pages.

### **Land Use/Land Cover (LU/LC)**

Land use cover along the Assabet River is extremely varied but with GIS each parcel is identified, stored and routinely updated in the Massachusetts state government database. For the land use cover analysis I created a buffer around the Assabet River extending 500 meters on either side for a total area of 55,156 Km<sup>2</sup>. Next I manipulated the LU/LC data for the entire state of Massachusetts to include only the parcels that are within the Assabet River buffer (Table 2.2 and Figure 2.2). There are 26 different types of land use covers ranging in function from natural habitat, e.g. brushland/successional and forested wetland to urban habitat, e.g. high-density residential and commercial space. The land use cover with the most area is forest with 19,385 Km<sup>2</sup>, which is 35.15% of the total area of the Assabet River buffer. Other prominent natural habitats are non-forested wetland with 4,338.20 Km<sup>2</sup> or 7.87%, forested wetland with 3,102.35 Km<sup>2</sup> or 5.62% and water with 3,224.63 Km<sup>2</sup> or 5.85%. This buffer zone also includes the Assabet River National Wildlife Refuge, which covers an area of 9,024 Km<sup>2</sup>. Of the 26 types of land use covers that fall within the Assabet River buffer I chose thirteen that I thought highly impact the water quality of the Assabet River. The National Water Quality Inventory: 2000 Report [70] states that the leading sources of impaired waters in The United States are agricultural and urban land use cover, also known as non point sources (NPS). Agriculture accounts for 48% of pollution in U.S. rivers reported as impaired in 2000

[71]. In addition to the Assabet River buffer I also created a 0.5 Km<sup>2</sup> buffer around six sites that I chose for the GIS analysis (Figure 2.3). The buffers around these six sites include 22 (Table 2.3) of the 26 (Table 2.2) total land use covers found in the entire Assabet River Buffer. Both Figure 2.4 and Table 2.3 list thirteen high impact land use covers with one difference in each. Figure 2.4 lists waste disposal as a high impact land use cover because it is found within the Assabet River buffer but not within any of the site buffers. Table 2.3 lists 13 high-impact land use covers found within the six site buffers including urban public/institutional because it is found within three of the site buffers. Table 2.3 does not list not waste disposal because it is not found in any of the site buffers I selected for the Assabet River. The LU/LC within the 55,156Km<sup>2</sup> Assabet River buffer is diverse but I will go into more detail comparing and contrasting the LU/LC in the six GIS site buffers and describe how this might negatively impact the water quality of the Assabet River.

For the land use cover analysis I chose six locations along the Assabet River (Figure 2.3). These six locations are not the same as the water sample collection sites of 2010 and 2011. These locations were chosen to resemble the locations of the water samples collected in 2010 and 2011 while representing the entire length of the river. The six locations in Figure 2.3 begin in the bottom left corner near the Westborough WWTP and continue in a northeasterly direction toward the final location in the town of Concord. The first two sites represent locations between WWTPs. Site one is between the Westborough and Marlborough WWTPS and site two is between the Marlborough and Hudson WWTPs. Sites three and five are downstream of two WWTPs-Hudson and Maynard-and site four is upstream from the Maynard WWTP. Site six lies near the end of

the river where all water eventually flows creating a location representing a mixture of all the effluents discharged upstream. Even though the six GIS sites are not the same as the sites where water was collected for the 2010 and 2011 exposure studies I performed an in depth analysis of how land use cover and may impact water quality.

I created a 0.5 Km<sup>2</sup> rectangular buffer around the six locations to capture the land use covers around each site and separated the land use cover types into high impact and low impact for each site (Tables 2.3, 2.4 and Figure 2.5). The land use cover composition of these six buffers varies widely. A map displaying two of the sites, sites 4 and 5, can be seen in Figure 2.4. Site four has the largest area of high impact land use cover at 0.3775 Km<sup>2</sup> or 83.6% and the smallest area of low impact land use cover at 0.0751 Km<sup>2</sup> or 16.59%. Site five has the smallest area of high impact land use cover at 0.0670 Km<sup>2</sup> or 14.80% and the largest area of low impact land use cover at 0.3857 Km<sup>2</sup> or 85.20%. The buffer around site four has seven of the thirteen high impact land use covers including industrial, commercial, urban public/institutional, as well as four out of the five categories of residential including high, medium, low and multi-family. In contrast site five has only three high impact land use covers including industrial, mining and commercial. Sites four and five are interesting to compare because they are drastically different from one another but next I will detail how land use cover data of some of the GIS sites compare to the expression data gathered from the exposure experiments conducted in 2010 and 2011.

The six sites chosen for the GIS analysis are not the same as the locations chosen for water sample collection in 2010 and 2011, therefore I cannot make definitive conclusions but will extrapolate from the information I have. GIS site five is closest to

the site where the Maynard effluent water sample was taken for the exposure experiment conducted in 2011. When looking at the expression levels of Cytochrome P450 1A (CYP1A1) from that experiment there is no correlation with expression levels and land use cover. The expression levels for the Maynard effluent are relatively high compared to the laboratory water whereas its counterpart GIS site five has the lowest percent of high impact land use cover at 14.80%. The CYP1A1 expression data from the Westborough effluent is among the lowest where as its counterpart GIS site one has the second highest percentage of high impact land use cover at 63.04%. I therefore conclude from the biological assays and GIS land use cover analyses that land use cover in 2010 and 2011 did not affect water quality as it pertains to CYP1A1 expression in male Medaka livers. Even though distinct correlations were not seen from these sets of analyses GIS is still comprehensive and useful tool for the aquatic toxicology field.

### **Discussion**

GIS is a unique and comprehensive tool that can reveal large amounts of information about water quality when combined with chemical and biological analyses. In the limited scope of the present research no significant correlations were found. There are several factors that could account for this including; river flow, dilution and mixing, size and shape of buffers created around sites, land use cover designation and locations chosen for analyses. The results included in this thesis may not tell us exactly what is happening along the Assabet River but I believe the analyses are still relevant and useful. I believe that these same tools can be used in the future and with the appropriate data input we can gain meaningful information in the fields of environmental science and water quality.

**Table 2.1 Thirty-year population change of the nine towns along the mainstem of the Assabet River.**

	Year				
	1980	1990	2000	2010	
Town	Population				Change
<b>Acton</b>	17,672	17,872	20,331	21,924	4,252
<b>Berlin</b>	2,224	2,293	2,380	2,866	642
<b>Concord</b>	16,455	17,076	16,993	17,668	1,213
<b>Hudson</b>	17,369	17,233	18,113	19,063	1,694
<b>Marlborough</b>	31,550	31,813	36,255	38,499	6,949
<b>Maynard</b>	9,822	10,325	10,433	10,106	284
<b>Northborough</b>	10,741	11,929	14,013	14,155	3,414
<b>Stow</b>	5,144	5,328	5,902	6,590	1,446
<b>Westborough</b>	13,261	14,133	17,997	18,272	5,011
<b>Total</b>	<b>124,238</b>	<b>128,002</b>	<b>142,417</b>	<b>149,143</b>	<b>24,905</b>

**Table 2.2 Distribution of the different Land Use/Land Cover (LU/LC) types in the 500m buffer around the Assabet River.**

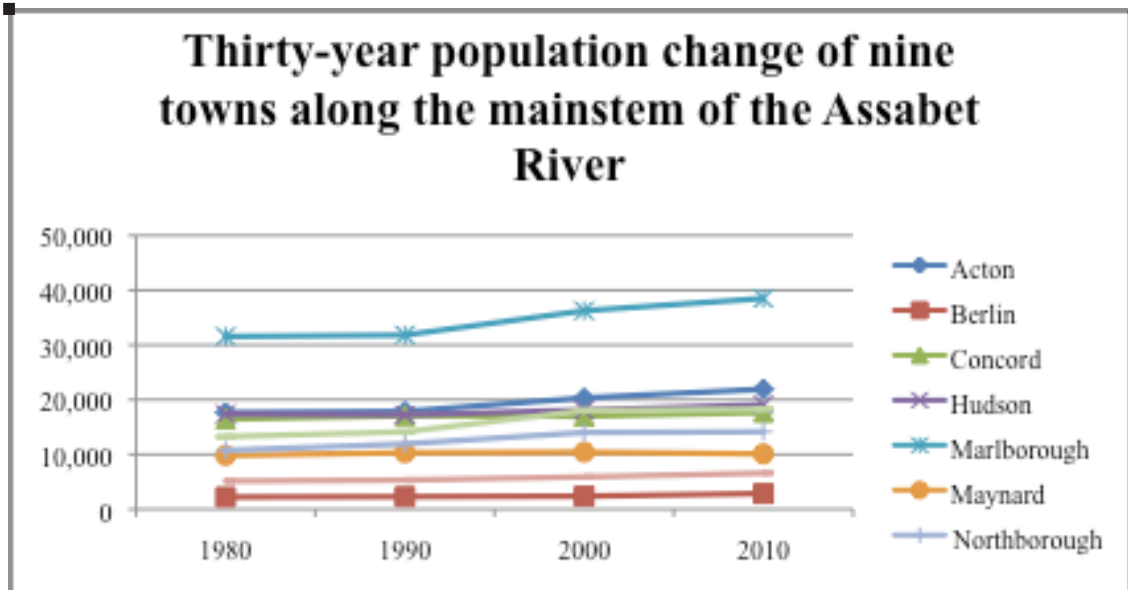
Land Use/Land Cover Type	Land Area Km <sup>2</sup>	% of Total Land Area
Cropland	1761.24	3.19
Pasture	588.84	1.07
Forest	19385.32	35.15
Non-Forested Wetland	4338.20	7.87
Mining	310.29	0.56
Open Land	896.41	1.63
Participation Recreation	556.29	1.01
Multi-Family Residential	1922.45	3.49
High Density Residential	1036.58	1.88
Medium Density Residential	5150.83	9.34
Low Density Residential	4560.35	8.27
Commercial	2489.29	4.51
Industrial	1374.98	2.49
Transitional	176.77	0.32
Transportation	554.25	1.00
Waste Disposal	351.68	0.64
Water	3224.63	5.85
Powerline/Utility	78.98	0.14
Golf Course	1176.83	2.13
Urban Public/Institutional	729.28	1.32
Cemetery	115.83	0.21
Orchard	229.98	0.42
Nursery	90.98	0.16
Forested Wetland	3102.35	5.62
Very Low Density Residential	915.11	1.66
Brushland/Successional	38.37	0.07
<b>Total</b>	<b>55156.10</b>	<b>100.00</b>

**Table 2.3 Percentages of Land use/Land cover types in the 0.5 Km<sup>2</sup> rectangular buffer around each of the six sites chosen for GIS analysis.**

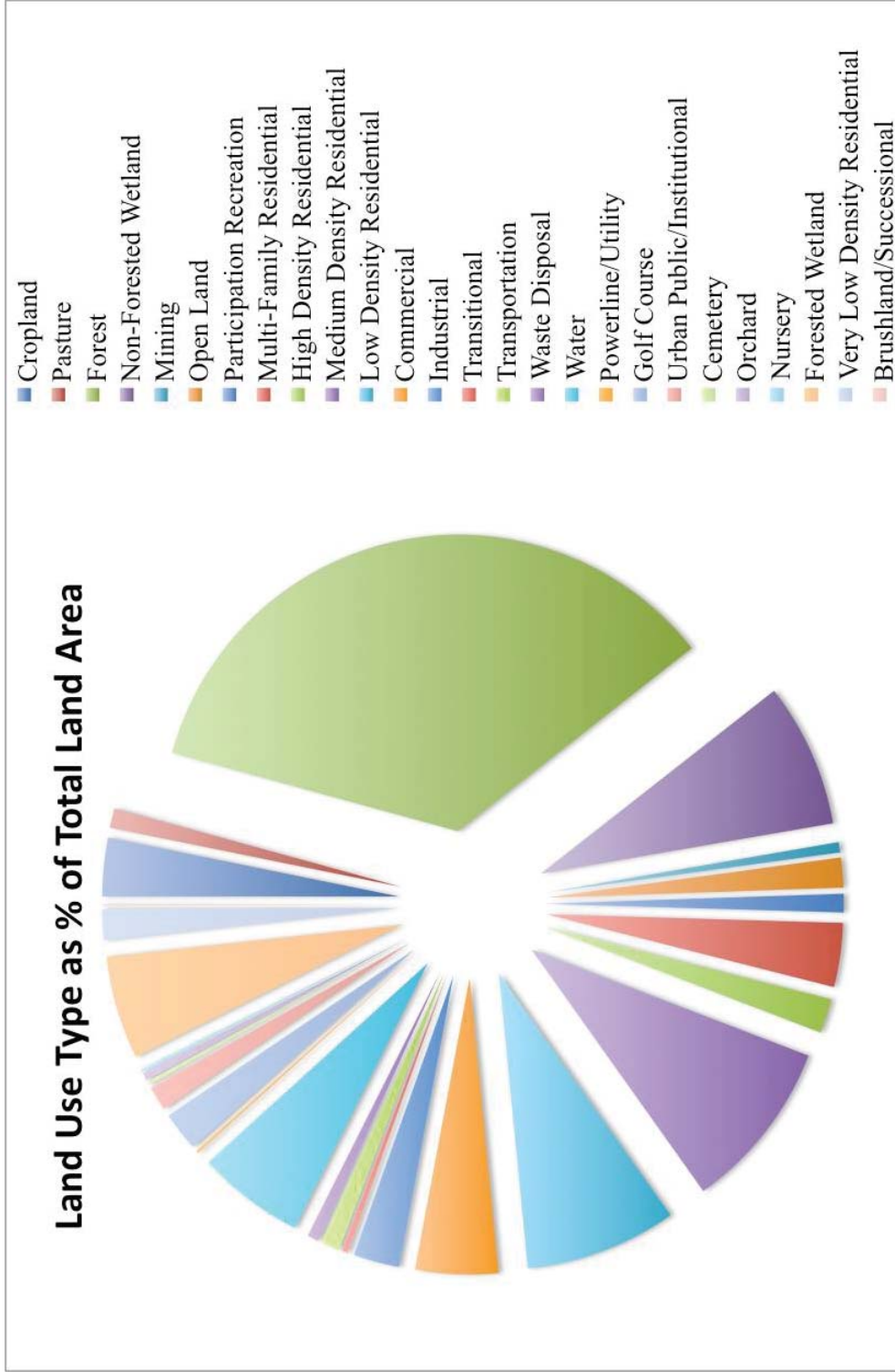
Land Use/Land Cover Type	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
<b>High Impact</b>						
<b>Golf Course</b>	52.22%	0	0	0	0	0
<b>Industrial</b>	0	0.13%	0	3.14%	6.32%	0
<b>Mining</b>	0	0	0	0	3.98%	0
<b>Commercial</b>	1.44%	0.86%	0	21.79%	4.51%	0
<b>Cropland</b>	0	0	2.41%	0	0	13.55%
<b>Orchard</b>	0	0	10.43%	0	0	0
<b>High Density Residential</b>	0	0	0	31.62%	0	0
<b>Medium Density Residential</b>	9.01%	18.45%	0	11.91%	0	13.02%
<b>Low Density Residential</b>	0	14.74%	8.02%	0.33%	0	21.02%
<b>Very Low Density Residential</b>	0	0	0	0	0	0.57%
<b>Multi-Family Residential</b>	0	0.75%	0	14.03%	0	0
<b>Urban Public/ Institutional</b>	0.38%	8.88%	0	0.60%	0	0
<b>Transportation</b>	0	0.07%	0	0	0	0
<b>High Impact Total</b>	<b>63.04%</b>	<b>43.89%</b>	<b>20.85%</b>	<b>83.41%</b>	<b>14.80%</b>	<b>48.15%</b>
<b>Low Impact</b>						
<b>Forest</b>	25.89%	30.59%	41.73%	9.06%	65.34%	28.11%
<b>Forested Wetland</b>	0.57%	8.35%	13.12%	1.22%	0	7.98%
<b>Non-Forested Wetland</b>	7.42%	15.36%	17.34%	1.72%	8.84%	11.45%
<b>Pasture</b>	0.86%	0.11%	3.05%	0	0	0.60%
<b>Water</b>	2.21%	1.70%	3.91%	1.39%	2.67%	3.69%
<b>Participation Recreation</b>	0	0	0	2.14%	6.72%	0
<b>Transitional</b>	0	0	0	1.06%	0	0
<b>Open Land</b>	0	0	0	0	1.63%	0
<b>Brushland/Successional</b>	0	0	0	0	0	0.02%
<b>Low Impact Total</b>	<b>36.96%</b>	<b>56.11%</b>	<b>79.15%</b>	<b>16.59%</b>	<b>85.20%</b>	<b>51.85%</b>
<b>Total</b>	<b>100.00%</b>	<b>100.00%</b>	<b>100.00%</b>	<b>100.00%</b>	<b>100.00%</b>	<b>100.00%</b>

**Table 2.4 Total area of high impact and low impact land use/land cover (LU/LC) types in the 0.5 Km<sup>2</sup> rectangular buffer around each of the sites chosen for GIS analysis.**

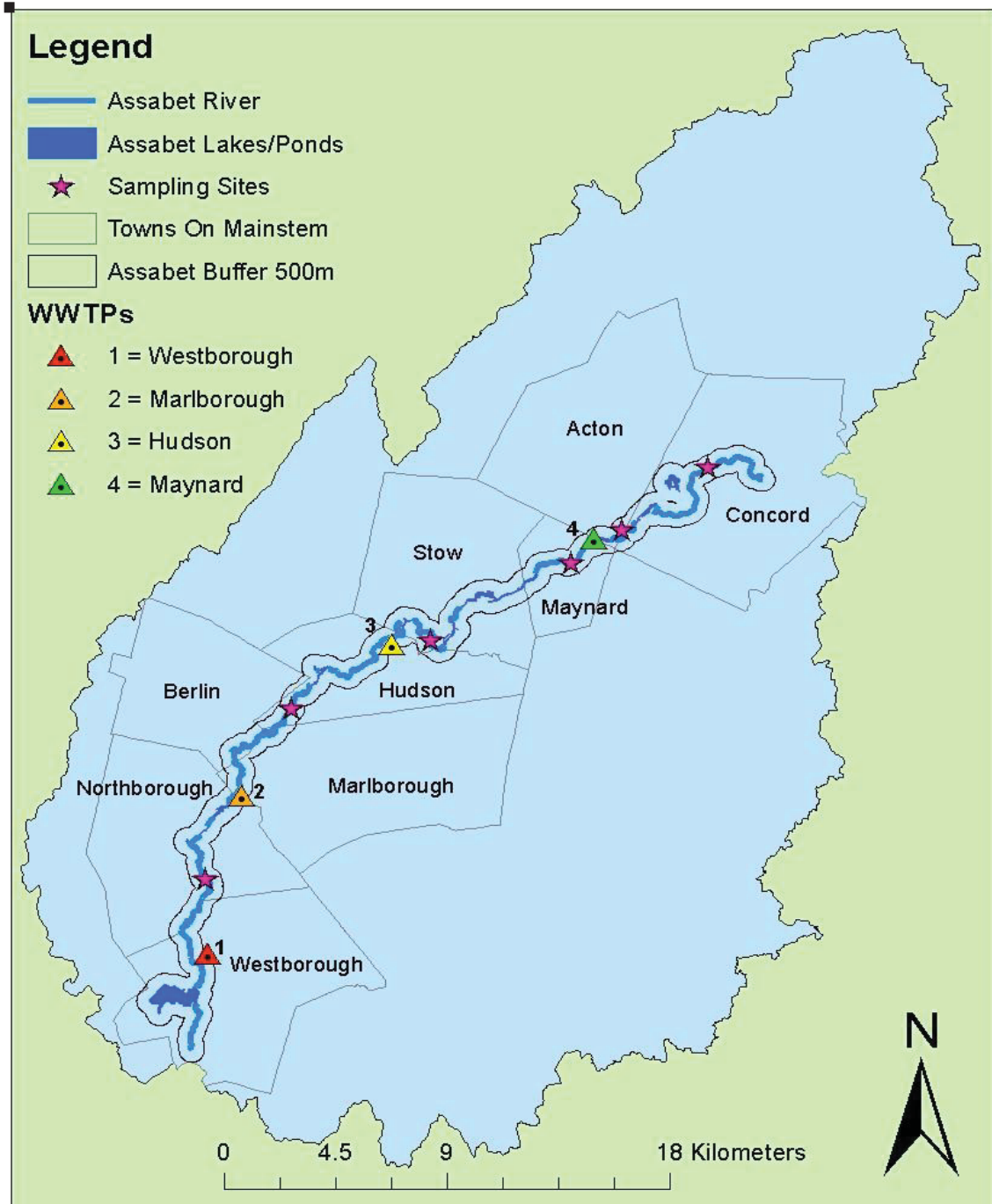
Land Use	Site					
	1	2	3	4	5	6
	Km <sup>2</sup>					
<b>High-Impact Land Use</b>	0.2854	0.1986	0.0363	0.3775	0.067	0.2179
<b>Low-Impact Land Use</b>	0.1673	0.2539	0.4164	0.0751	0.3857	0.2346
<b>Total</b>	<b>0.4527</b>	<b>0.4525</b>	<b>0.4527</b>	<b>0.4526</b>	<b>0.4527</b>	<b>0.4525</b>



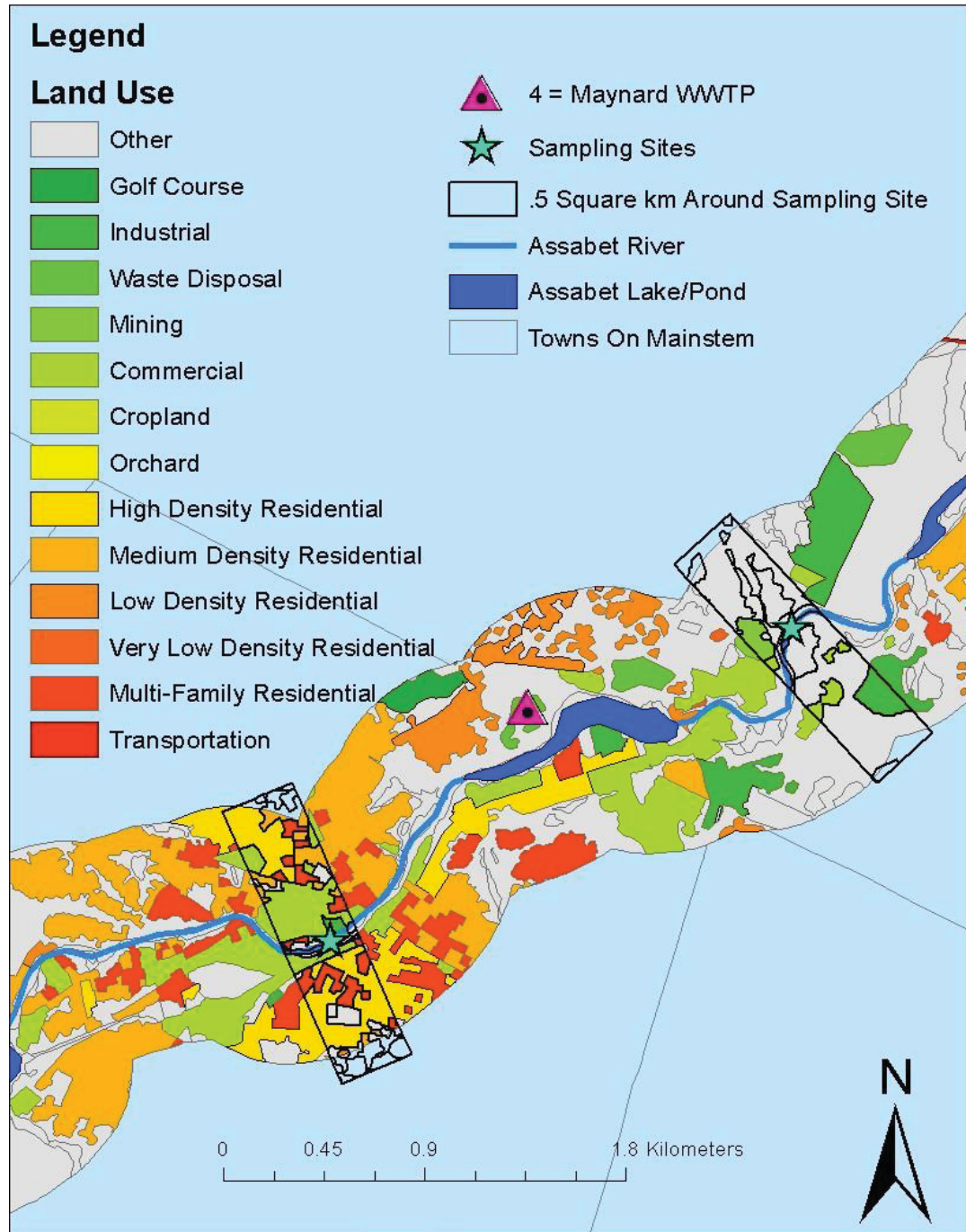
**Figure 2.1** Change in total population of nine towns along the mainstem of the Assabet River. Nine towns lie along the mainstem of the Assabet River and all of their populations have increased over the past 30 years.



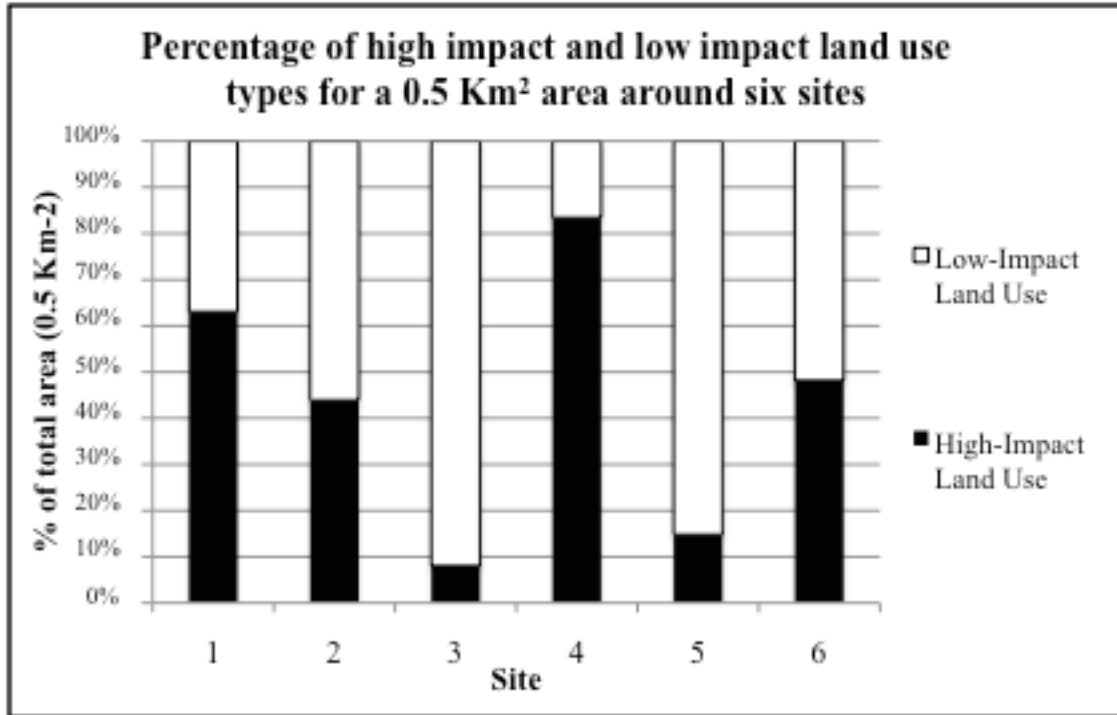
**Figure 2.2 Land use/land cover (LU/LC) types in the 500m buffer around the Assabet River.** There are 26 different LU/LC types in the 500 m Assabet River buffer. Forest is the largest LU/LC type at 35.15%. See Table X. for a complete list.



**Figure 2.3** Map of the Assabet River Watershed used for GIS analyses. This map identifies the nine towns along the mainstem, the four major WWTPs and six sites on the river chosen for Land Use/Land Cover analysis using GIS.



**Figure 2.4** Map of high and low impact Land Use/Land Cover (LU/LC) types in a 500m buffer zone around the Assabet River. The Assabet River buffer extends 500 m on either side of the river and the sample buffers (rectangles around stars) form a 0.5 Km<sup>2</sup> rectangular buffer around each sampling site. Colored (other than gray) LU/LC types highly impact water quality and gray LU/LC sites are considered to have a low impact on water quality. Location 4 (left star) has significantly larger high impact LU/LC types than site 5 (right star).



**Figure 2.5** Percentage of high impact and low impact land use/land cover (LU/LC) types in the buffers around the six GIS sites. Total percentage of high impact versus low impact LU/LC types in the 0.5 Km<sup>2</sup> rectangular area around each of the six sites chosen for GIS analysis.

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