Hydrographic Study of Wastewater Treatment Plant Effluent in the Royal and Cousins Rivers of Yarmouth, Maine

Report of Findings from the May 24 – 26, 2010 Study Period

FDA Technical Assistance and Training Project

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June 2013

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1.0 INTRODUCTION

1.1 Study Objectives

A hydrographic dye study of effluent from the Yarmouth Wastewater Treatment Plant (WWTP) was conducted on May 24-26, 2010 to assess the dilution, time of travel, and dispersion of effluent in the Royal River and Cousins River. Five cages filled with oysters were deployed at various locations (stations) along the anticipated path of the effluent to correlate the dye concentrations found at the cages with the indicator bacteria and viral findings in the oysters. Natural set soft-shell clams in the vicinity of three of the oyster cages were also assessed for these criteria.

The study objectives were to:
(1) determine the bacterial and viral conditions that could arise under a short term lapse in treatment and disinfection at the WWTP;
(2) determine the steady state bacterial conditions in the shellfish growing waters that could arise in the event of a long term elimination or lapse in disinfection;
(3) provide guidance to the Maine Department of Marine Resources (DMR) regarding WWTP closure zones based on dilution of WWTP effluent; and
(4) research the dilution level needed to achieve a sufficient reduction in viruses to ensure the safety of shellfish harvested in proximity to WWTPs as part of FDA's dilution guidance.

This was a follow-up to a study of the impact of the Yarmouth WWTP's effluent on shellfish resources in the Royal River that was conducted by FDA and DMR in 2002 (reference: "Hydrographic Study of Yarmouth Maine Waste Water Treatment Plant Effluent, Report of Findings from the August 17 – August 22, 2002 Study Period"). A new diffuser was installed at the Yarmouth WWTP since 2002, and FDA has since acquired new fluorometers and geospatial mapping technologies that allowed for a more comprehensive study to address some of the issues raised in the previous study report.

1.2 FDA Guidance on Establishing Closure Zones for WWTP Discharges

In consideration of Section II, Chapter IV @.03 E(5) (Prohibited Classification – Wastewater Discharges) of the National Shellfish Sanitation Program Model Ordinance, which notes that the determination of the size of a prohibited zone around a WWTP outfall shall include "the wastewater's dispersion and dilution, and the time of waste transport to the growing area where shellstock may be harvested" (iii), FDA has provided guidance to state shellfish control authorities to size prohibited zones around WWTP outfalls according to the following scenarios:

Scenario 1: In consideration of effluent discharged from a WWTP under **failure conditions** (such as a loss of disinfection), the prohibited zone should provide a sufficient amount of dilution to dilute the effluent discharged under failure conditions to the fecal coliform standard of 14 MPN/100 ml within the prohibited zone

OR

Scenario 2: In order to reduce the size of the prohibited zone, a conditionally approved zone may be operated **if** a factor of at least a 1000:1 dilution of effluent is achieved within the prohibited area to mitigate the impact of viruses, **and** there is a sufficient amount of time to close the conditional area to the harvesting of shellfish **before** the effluent discharged at the onset of a failure can travel to the boundaries of the prohibited zone

Note: the additional area beyond the prohibited zone to be closed under WWTP failure conditions should provide a sufficient amount of dilution to dilute the effluent discharged under failure conditions to the fecal coliform standard of 14 MPN/100 ml within the closed (due to failure) zone (consistent with Scenario 1).

Over the years, wastewater treatment technologies have improved. However, FDA has maintained a conservative position recognizing that a WWTP may still be subject to failure regardless of the type of treatment system used. FDA does recognize that with the advancement of technologies such as improved monitoring and alarm systems, it may be possible to operate a conditional area as outlined in Scenario 2 above. This allows additional shellfish growing areas to be harvested under certain conditions.

When a WWTP is operating normally, disinfection has been shown to be effective in reducing the coliform bacteria group (fecal coliform and total coliform) to levels below shellfish harvesting standards as can be seen in WWTP permit records kept in accordance with the Environmental Protection Agency (EPA) National Pollutant Discharge Elimination System (NPDES) Program. However, human enteric viruses such as noroviruses and hepatitis A virus are more resistant to disinfection and thus are not reduced to the same degree as the coliform bacteria group. In an effort to mitigate the risk of contaminating shellfish with viruses, FDA has recommended a 1000:1 dilution as described in Scenario 2 as the minimum zone of dilution needed when the WWTP is operating under normal conditions. Included in this report is an estimation of the 1000:1 dilution area for the Yarmouth WWTP based on the steady state dilution levels found at the cage stations along the path of the WWTP effluent and the first day (ebb tide excursion) dye results. However, adjustments to the 1000:1 dilution area found on the first day to account for lower or higher WWTP flow conditions and other considerations are also discussed.

1.3 Description of Yarmouth WWTP

Figure 1 shows a map of the Royal River with the five oyster sentinel stations and the Yarmouth WWTP's diffuser location. The Cousins River runs northwest of Station 3. Soft-shell clam resources are located in both the Royal and Cousins Rivers. The Yarmouth WWTP is located on the southern shore of the Royal River at 82 Princess Point Road in Yarmouth. The WWTP underwent an upgrade in 1992. The average daily flows from the WWTP are in the range of approximately 0.5 – 2.0 million gallons a day (MGD). The monthly average flow discharge limitation in the WWTP's Maine Pollutant Discharge Elimination System (MEPDES) Permit (#ME0100765) is 1.31 MGD for secondary treated wastewaters. The level of 1.31 MGD is the only flow discharge limitation provided in the permit. Wastewater is disinfected year round with sodium hypochlorite in three chlorine contact tanks and dechlorinated using sodium bisulfite.

The diffuser is designated with a yellow marker in Figure 1. The diffuser is located on the southern shore of the Royal River approximately 1.1 nautical miles (nm) above the confluence of the Royal and Cousins Rivers. Since FDA conducted its previous study in 2002, the Yarmouth WWTP relocated and retrofitted its 16 inch diameter single-port outfall with a submerged multi-port outfall (diffuser). The new outfall pipe measures 20 inches in diameter and at the end of the pipe the new diffuser has seven ports each measuring 6 inches in diameter spaced at 10 feet on-center. The new outfall pipe extends out in the deepest portion of the navigation channel. Based on CORMIX modeling conducted by Wright-Pierce, it was estimated that the new diffuser would improve dilution ratios of the effluent by levels of 3:1 – 8:1 or 20:1 – 100:1.

1.4 General Description of Study Design

Based on the results of FDA's previous dye study in 2002, five oyster cages (custom-made by Chesapeake Bay Oyster Co., Wake, VA) filled with 3-inch local oysters and equipped with WET Labs submersible fluorometers (WET Labs, Inc., Philomath, OR) were placed at various distances along the path of the effluent plume on May 20, 2010, prior to the hydrographic dye study that took place from May 24 – 26, 2010. Stations (Cages) 1, 2, 3, 4, and 5 were approximately 0.3, 0.9, 1.5, 2.4, and 3.6 miles away from the WWTP diffuser, respectively. The cage closest to the WWTP's diffuser and the cage farthest away were also equipped with CTDs to monitor conductivity/salinity, temperature, and depth/pressure during the course of the dye study. The cages remained in the water until June 2, 2010 to give the oysters at least two weeks of exposure time to the WWTP effluent.

Also prior to the May 24 – 26, 2010 dye study, a drogue study was conducted on May 21, 2010 to determine the current velocity, direction, and dispersion in the Royal River and to ascertain if the oyster cages placed along the path of the effluent plume from the Yarmouth WWTP were positioned properly to maximize the oysters' exposure to viruses. The drogue study also provided information about tidal cycles in the Royal River to help plan the timing of the dye study. Members of Environment Canada (EC), Jeff Stobo and Patrice Godin, provided GPS-tagged drogues and assisted with the drogue study and the ensuing hydrographic dye study.

The dye for the comprehensive study was injected over half a tidal cycle (12.4 hours) and remained in the Royal River system for at least three days. Boat tracking with towed WET Labs fluorometers was conducted to find the edges of the dye plume during daylight hours, in addition to the 24/7 dye readings recorded by the cage-attached submersible fluorometers. The submersible fluorometers were collected from the cages on May 28, 2010, when dye readings were falling below detectable levels, but the cages were left in for several more days to allow the oysters additional exposure time to viruses in the effluent.

In addition to the oyster cages placed by FDA in the Royal River during the study, representatives from Spinney Creek Shellfish, Inc. (hereafter referred to as "Spinney Creek") collected soft-shell clams at three sampling stations in the Royal River. Spinney Creek tested the clams for fecal coliforms (FC) and male-specific coliphage (MSC) and FDA's Gulf Coast Seafood Laboratory (GCSL) in Dauphin Island, AL tested the clams for norovirus genogroups I and II (NoV GI and GII).

The oysters from the five FDA cages (stations) were also shipped to FDA's GCSL to test for the presence of FC, *E. coli* (EC), MSC, and NoV GI and GII. Samples taken from the influent and final effluent flows at the Yarmouth WWTP were also analyzed for FC, EC, and MSC to compare with the levels found in the oysters at each of the cage stations. The results of these microbiological analyses were compared with the level of dilution of dye found at each of the cages to determine a relationship between corresponding dilution from the WWTP effluent and viral impacts on shellfish and the level of dilution is needed to sufficiently minimize those impacts.

2.0 METHODS

2.1 Dye Standard Preparation and Fluorometer Calibration

The dye tracer used in this study was Rhodamine WT, purchased from the Keystone Aniline Corporation, with a specific gravity of approximately 1.12 (20% as dry dye). Ten (10) standards were prepared from the stock solution of Rhodamine WT dye and distilled water by serial dilution, ranging from 100,000 parts per million (ppm) to 0.1 parts per billion (ppb).

The Rhodamine WT dye was detected and its concentrations in the Royal and Cousins Rivers were obtained using a combined total of seven fluorometers. Five of these were WET Labs FLRHB submersible fluorometers (WET Labs, Inc., Philomath, OR) that were attached to the shellfish cages deployed at stations along the anticipated path of the effluent throughout the course of the study. One was a WET Labs FLRHRT fluorometer that was pulled behind a boat and used for tracking the dye on the ebb tide for each day of the study. The other fluorometer was a Turner 10-AU digital field fluorometer (Turner Designs) that was also used for boat tracking of the dye on the ebb tide of each day.

The dye standards were used to develop calibration curves for FDA's WET Labs FLRHRT-586 tracking fluorometer and the five submersible fluorometers – WET Labs FLRHB units 585, 913, 915, 1730, and 1731. With the subtraction of background fluorescence levels in the Royal River, these curves were used to calculate part per billion (ppb) levels of dye based on the WET Labs' measured fluorescence units (FUs). Background readings were captured on the day prior to the study, May 23, 2010.

The y-intercept of the calibration curve was adjusted so that a "0.1 ppb" result read as a perfect "0.1" on the curve. The slope and x-axis values for the curve remained the same, but this adjustment caused a slight addition of error (5-10% error) to the higher concentrations on the curve, such as 10 ppb and 100 ppb. However, higher accuracy at the lower end of the curve, 0.1 ppb, is more vital in order to optimize sensitivity in detecting the dye at low concentrations, as important data tends to fall within the 0.1-1 ppb range during FDA dye studies. Using a calibration curve adjusted in this manner is necessary when converting raw FU readings to ppb values if sensitivity in the 0.1-1 ppb range is critical for the study.

The Turner Designs 10-AU digital field fluorometer was calibrated and verified using the 100, 10, 1, and 0.1 ppb standards. Prior to calibration, the basic operating level was set using the sensitivity adjustment knobs and a 1 ppb standard at medium scale. The field fluorometer was flushed and

blanked during calibration with river water followed by distilled water flushes. The calibration was conducted according to the procedure in the fluorometer user's manual, but using a 1 ppb as the reference standard (rather than 10 ppb) and river water (rather than distilled water) as the calibration blank. For the "Subtract Blank" option, "Yes" was selected. The linear operation of the fluorometer was then checked using the standard solutions.

2.2 Drogue Study

Approximately fifty oranges and grapefruits, three FDA "winged drogues" constructed from PVC pipe and aluminum sheet metal, and two GPS-tagged Environment Canada "winged drogues" were used on May 21, 2010 to assess the timing of tidal cycles (i.e., slack high/start of ebb tide), tidal velocity, and the influence of wind to estimate the velocity and direction of the effluent leaving the WWTP diffuser. The fruit drogues were released on the surface of the water, while the aluminum sheets of the winged drogues were suspended to a depth of approximately 1 – 2 feet by the PVC pipe in order to capture the tidal flow and be less influenced by surface winds.

A portion of the oranges were thrown in a horizontal line near the outfall prior to the turning of the tide from flood to ebb tide. The drogues were not marked, but the time of release for each drogue was recorded. Additional lines of orange and grapefruit drogues were dispatched at varying time intervals to assess how the shifting tide would impact the movement of the drogues. After the tide switched to ebb, the winged drogues were also dropped in proximity to the outfall boil, with a brief interval between each drogue drop. These drogues were then tracked along the anticipated path of the effluent plume, and the approximate direction and distance the drogues traveled were noted at various time points and were also recorded with Garmin GPS units.

2.3 Dye Injection

For the dye injection, a total of 3 Gallons of dye was injected at a constant rate into the Yarmouth WWTP effluent over a 12.4 hour period from 2:15 AM – 2:40 PM on May 24, 2010. To facilitate the pumping of dye, 3 gallons of deionized water was added creating a 1:2 dye dilution mixture (6 gallons total). A Masterflex model 7553-20 variable speed peristaltic pump (Cole-Palmer Instrument Co.) was used to withdraw the tracer dye solution from a large plastic holding bin, using Masterflex Tygon L/S-14 tubing. A pump head size 7014 was used with a constant pumping rate of 30.5 ml/min which was maintained at about 124 revolutions/minute (rpm) head speed. The tracer dye mixture was fed continuously into the final effluent following the chlorine treatment over the 12.4 hour injection period. The initial concentration of the dye in the effluent was determined using the WWTP's flow average over the course of the dye injection period.

2.4 Dye Tracing

The dye plume was followed during the beginning of the study on May 24, 2010 as it moved through the Royal River and Cousins River on ebb tide using FDA's WET Labs FLRHRT-586 fluorometer and Turner 10-AU fluorometer. The fluorometers were linked to Trimble GPS units operating with Terrasync software, and the GPS coordinates for the outer edges of the dye were located.

Two boats were used, with each instrument on a different boat. Officials from the Environmental Protection Agency (EPA) monitored and assisted with the study from a third boat. Dye readings were also taken on successive days (May 25 - 26) for high and low tides. Traverses were done on all the days of the study from north to south and east to west and vice versa, and dye readings were also recorded at each of the station locations (via boat and with the submersible fluorometers fixed to the stationary cage stations) to show changes in dye concentration and build-up with time at the fixed locations.

A five-point moving average was applied to the dye concentration data to smooth out any false high or low readings. Dilution was calculated by dividing the initial concentration of dye injected at the WWTP by the final (five-point moving average) concentrations detected in the estuary.

The fluorometer dye concentration readings (in fluorescent units) with the associated GPS readings were later downloaded and converted into ppb units using the calibration curve for WET Labs FLRHRT-586. The readings for the Turner 10-AU fluorometer were recorded in ppb units directly. The concentration values were then converted as color-coded maps in ArcGIS Desktop v. 10.0 to create color-coded maps representing the presence of different dye concentrations along the path of the effluent for each day of the study. The dilution values were calculated by dividing the initial concentration of dye in the effluent by the final concentration of dye in the estuary. The dilution values were also plotted in color-coded GIS maps using ArcGIS v. 10.0.

2.5 Dilution Analysis - Dye Readings from Submersible Fluorometers

The fluorescence readings recorded by the submersible fluorometers at each of the five stations were downloaded, converted to ppb using each fluorometer's calibration curve chart, and plotted in SigmaPlot alongside the CTD tidal depth curves for the period of the study.

A five-point moving average was applied to the dye concentration data to smooth out any false high or low readings in the data. Dilution was calculated by dividing the initial concentration of dye injected at the WWTP by the final (five-point moving average) concentrations detected in the estuary.

Since only a 12.4 hour dye injection was conducted, the superposition method (Kirkpatrick, 1993) was used to estimate the steady state condition for dye at each of the cage stations using data collected from May 24, 2010 – May 28, 2010. In the 2002 Yarmouth study and other past studies, FDA would typically conduct a 2 – 3 day injection of dye to determine the build-up of WWTP effluent in an estuary system and to determine the steady state condition, in which the rate of effluent flowing into a system is equal to that being flushed out by tides. However, Kirkpatrick (1993) demonstrated using the superposition principle that a shorter dye injection period (24.8 hours or a single tidal day) could be used and the steady state condition estimated if remaining dye in the system on the second tidal day after an injection is added to the dye detected on the first tidal day, and if the remaining dye detected on the third tidal day is added to the dye found on both the first and second tidal days, and so on. FDA has successfully employed

the superposition method, even with only a half tidal day (12.4 hour) injection, and used this method in the 2010 Yarmouth study to save time and resources.

For the day of the injection, May 24, 2010, the low tide concentrations, peak 1 hour concentrations, and average concentrations of dye were plotted. For the second day of the study, May 25, 2010, dye still remained in the system, so this remaining dye level (including low tide concentrations, peak 1 hour concentrations, and average concentrations) was added to the levels detected on day 1 and plotted. Following the superposition principle, remaining dye levels found in the system on successive tides were used to determine the steady state condition at each cage station.

2.6 Microbiological Analysis of Wastewater and Oysters

Shellfish Sentinels at Station Locations

Local oysters (Glidden Point Oyster Sea Farm) approximately 3 inches in size and aquaculture grown in the Damariscotta River were used as shellfish sentinels at each of the 5 station locations. The oysters were depurated and tested for microbial indicators by Spinney Creek prior to deployment. A total of 300 oysters were used in the study, with some oysters used as controls. There were approximately 50 oysters per cage for each of the 5 cages. Within each cage, there were 5 bags of oysters with 10 oysters each. One bag was tied to each corner of the cage, with the fifth bag in the middle of the cage. The cages were weighed down with cement and were stationary on the bottom of the estuary. The oysters were deployed on May 20, 2010 and were recovered and tested two weeks later on June 2, 2010.

In addition to the oyster cages placed by FDA in the Royal River during the study, Spinney Creek collected natural set soft-shell clams at three sampling stations in the Royal River. The soft-shell clams were collected near the oyster sentinel cages closest to the WWTP diffuser – Stations 1, 2, and 3.

Indicator Microorganisms

FC and EC densities in the shellfish and in the WWTP influent and effluent were determined using a conventional five-tube, three-dilution MPN procedure. In the case of the shellfish, the procedure was done with minimal modifications to the FDA *Bacteriological Analytical Manual* (BAM) and American Public Health Association (APHA) (1970) recommended procedures for the examination of shellfish. Modifications to this procedure included blending of the shellfish meats and liquors without dilution buffer; this was necessary due to the multiple microbial analyses performed on each shellfish sample. Following homogenization, a 1:10 dilution of homogenate (10 g) was prepared with phosphate-buffered solution (PBS). Ten milliliters of this dilution, a 1-g equivalent, was transferred to five tubes of 10 ml of double-strength lauryl tryptose broth (LST; Difco Laboratories, Sparks, MD). One-milliliter aliquots (0.1-g equivalent) were also transferred to five tubes of single-strength LST, while five 1-ml aliquots of a 1:100 dilution were also transferred to single-strength LST. Presumptive positive tubes were confirmed for FC and EC using EC-MUG (Difco, Sparks, MD) medium.

MSC densities were determined by using a modified double-agar-overlay method initially described by Cabelli (1988); the *E. coli* strain HS(pFamp)R (ATCC 700891) was utilized as the bacterial host strain.

MSC testing was conducted by Spinney Creek on natural set soft-shell clams taken from beds near Stations 1, 2, and 3. Spinney Creek employed the same MSC method as described above (NSSP, 2009). Spinney Creek also plotted standard error bars for the triplicate data gathered for the MSC findings. In addition, Spinney Creek examined method and biological variation by plotting the mean of the replicates vs. the coefficient of variation. The hyper-accumulation period of the clams at each station was plotted for comparison as well.

Virus concentration and RNA extraction

For the extraction method, 5 to 10 whole oysters were washed and shucked, and the digestive diverticula from 5 to 10 oysters were removed to obtain a total of 25 g of sample. An aliquot of non-human calicivirus (San Miguel sea lion virus, serogroup 17 [SMSV-17]) was added as an extraction control prior to homogenization of the digestive diverticula with 7x H_2O (7 volumes of 25 g). A total of 105 g of the homogenate was added into a tared 250-ml centrifuge bottle.

Conductivity was measured using a 4-ml aliquot of the homogenate (model ARH1; Myron L Company, Carlsbad, CA), and the 105-g homogenate was adjusted to less than 2,000 µS. Viruses were adsorbed onto the particulate by adjusting the pH to 4.8 ± 0.3 and then shaken on an orbital shaker (200 rpm) for 15 min at room temperature. After the absorption step, samples were then centrifuged for 20 min at 2,000 x *g* at 4°C; following centrifugation, the supernatant was discarded. The pellet was eluted with 105 ml of 0.75 M glycine-0.15 M NaCl, and the pH was adjusted to 7.5 ± 0.2, followed by an additional elution with 52.5 ml of 0.5 M threonine-0.15 M NaCl. The eluates were combined and precipitated with 8% polyethylene glycol (PEG)-0.3 M NaCl and incubated for 3 h or overnight at 4°C. Precipitates were spun, and the pellet was resuspended in 12 ml of tissue culture-grade phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2PO_4 per liter).

Samples were then extracted, first with 12 ml of chloroform by vortexing for 1 min, and then centrifuged at 1,700 x *g* for 30 min at 4°C. The upper aqueous phase was transferred to a clean, 50-ml conical tube. The remaining portion was extracted with 6 ml of 0.5 M threonine-0.15 M NaCl and centrifuged as previously described. The two aqueous phases were combined and precipitated with 8% PEG-0.3 M NaCl for 3 h or overnight at 4°C. Following precipitation, samples were centrifuged at 20,800 x *g* for 15 min at 4°C and pellets were extracted for RNA, utilizing 6 M guanidium isothiocyanate as a lysis solution and the RNeasy minikit (Qiagen, Valencia, CA). Extracted RNA was tested by real-time PCR as described below.

Real-time RT-PCR for NoV GI and GII

Positive controls used for NoV GI and GII were *in vitro* RNA transcripts of sequences cloned from positive clinical samples previously identified as NoV (see Burkhardt, et al., 2006). Primers and probes for NoV GI and GII targeted the most conserved region of the open reading frame 1 (ORF1)-ORF2 junction. Real-time reverse transcription (RT)-PCR for detection of NoV GI and NoV GII with an RNA IAC was performed in a 25-µl reaction volume by using a one-step RT-PCR kit (Qiagen). The primer concentrations for the NoV targets were 300 nM each, and the

concentrations for the IAC primers (46F and 194R) were 75 nM each. The 5' nuclease probe concentrations for NoV and the IAC target were 100 and 150 nM each, respectively. The final concentration of $MgCl_2$ in the real-time RT-PCR was 4 mM. Thermal cycling was run using the SmartCycler II system with the following conditions: 50°C for 3,000 s and 95°C for 900 s followed by 50 cycles of 95°C for 10 s, 53°C for 25 s, and 62°C for 70 s. Fluorescence was read at the end of the 62°C elongation step. Default analysis parameters were used, except that the manual threshold fluorescence units were set to 10. Samples positive with the initial primer and probe sets for NoV GI and/or NoV GII were subjected to a secondary detection assay. Amplification of the original RNA extract was performed with primers from the B region by conventional RT-PCR (see Table 1 in DePaola, et al., 2010). Amplification of a second region of the genome is non-contiguous to the first and serves as an indication that the RNA was not degraded. Another purpose is the production of a larger cDNA fragment that is needed to distinguish viral strains.

Hepatitis A Virus

The positive control used for HAV was the vaccine strain HM175/18f (subgenotype 1B), propagated in-house by utilizing the FRhK-4 cell line. Real-time RT-PCR for the detection of HAV with an RNA IAC was performed in a 25-µl reaction volume by using a one-step RT-PCR kit (Qiagen). The primer concentrations for HAV and the IAC were 300 nM and 75 nM, respectively, while the 5' nuclease probe concentrations for HAV and the IAC targets were 200 and 150 mM, respectively. The final concentration of $MgCl_2$ in the RT-PCR was 4 mM. Thermal cycling was run using the SmartCycler II system with the following conditions: 50°C for 3,000 s and 95°C for 900 s followed by 50 cycles of 95°C for 10 s, 53°C for 25 s, and 64°C for 70 s. Fluorescence was read at the end of the 64°C elongation step. Default analysis parameters were used except that the manual threshold fluorescence units were set to 10. Samples showing amplification with the initial primers/probe set were also subjected to a secondary detection assay for the same purpose as described for NoV above by using CDC nested primers and conventional RT-PCR.

SMSV-17

The positive control used for San Miguel sea lion virus 17 (SMSV-17) was propagated in-house by utilizing the Vero cell line. Real-time RT-PCR was utilized for the detection of SMSV-17 (the extraction control virus) with an RNA IAC in a 25-µl reaction volume by using a one-step RT-PCR kit (Qiagen). The primer concentrations for SMSV-17 and the IAC were 300 nM and 75 nM, respectively, while the 5' nuclease probe concentrations for SMSV-17 and the IAC targets were 200 and 150 mM, respectively. The final concentration of $MgCl_2$ in the RT-PCR was 7 mM. Thermal cycling was run using the SmartCycler II system under the following conditions: 50°C for 3,000 s and 95°C for 900 s followed by 45 cycles of 94°C for 10 s, 62°C for 20 s, and 72°C for 40 s. Fluorescence was read at the end of the 72°C elongation step. Default analysis parameters were used except that the manual threshold fluorescence units were set to 10.

Adenovirus. The positive control used for Adenovirus (AdV) was serotype 41 isolated from a clinical stool sample, propagated in-house by utilizing the A-549 cell line. Real-time PCR for the detection of AdV was performed in a 25-mL reaction volume by using Platinum TAQ DNA Polymerase (Life Technologies, Grand Island, NY) as previously described with slight modifications (Woods). A DNA IAC utilizing the 46F and 194R primers and the TxRed-labeled

probe as previously described was added with final primer and probe concentrations of 0.75 mM and 1.5 mM, respectively (DePaola). Cycle parameters were slightly adjusted as follows: 95°C for 120 s followed by 50 cycles of 95°C for 3 s, 53°C for 10 s, and 65°C for 70 s. AdV primers and probe were previously described with slight modifications to the probe (Heim). The probe was FAM-ZEN labeled as a fluorescent dye on the 5' end and an Iowa Black quencher dye labeled on the 3'end. Fluorescence was read at the end of the 72°C elongation step. Default analysis parameters were used except that the manual threshold fluorescence units were set to 10.

3.0 RESULTS

3.1 Drogue Study

The orange and grapefruit drogues were heavily influenced by wind and tides and were quickly pushed onto the shore. However, the aluminum sheet metal of the winged drogues was suspended farther beneath the surface of the water, about 1 foot down, and therefore the winged drogues traveled in the direction of the ebb tide. Figure 2 shows the direction and speed of travel of the winged drogues as recorded by the internal Garmin GPS. The path the drogues traveled is marked with small purple points. As noted in the figure, at 7:30 AM on the day of the drogue study (May 21, 2010), the drogues were traveling at a speed of approximately 50 ft/minute or 0.57 miles/hour.

3.2 Background Readings

Background levels of fluorescence units (FUs) for the WET Labs FLRHRT-586 tracking fluorometer were measured as 81 - 89 FUs on average. These are normal background levels for an estuary system evaluated with the FLRHRT-586 fluorometer and are not considered indicative of excessive background levels, as are often seen in areas with high industrial activity. The background level was subtracted from the fluorescence readings during the dye studies.

For the Turner 10-AU fluorometer, water from the Royal River was used to calibrate the instrument, such that background levels were read as negative values or values below the 0.01 ppb limit of sensitivity of the Turner fluorometer.

3.3 Dye Injection

The dye injection began around 2:15 AM on May 24, 2010 and continued for 12.4 hours. Records from the Yarmouth WWTP showed that the average flow for that period of time was 0.60 MGD, which is a low to normal flow level for the dry weather conditions that day. According to NOAA's National Weather Service (http://water.weather.gov/precip/), on May 19 and 20, 2010, about 4 - 5 days prior to the study, approximately 1 – 2 inches of rain fell in the Yarmouth area. Rainfall recorded at the Yarmouth WWTP falls below this range with 0.88 inches recorded on May 19, 2010. However, rainfall was not recorded or observed during the study itself. For comparison, the average WWTP flow rate during the original dye study in August 2002, which was also conducted during dry weather, was a nearly identical 0.56 MGD.

Based on this flow out of the WWTP and using the concentration of dye in the jug (approximately 100,000,000 ppb) and the flow out of the jug (30.5 ml/min or 11.6 gal/day), it was determined

using a mass balance approach that the concentration of dye flowing out of the WWTP's outfall was 1924 ppb.

3.4 Travel Time

This study determined the extent of dye travel on the ebb tide of the first day of the study (May 24, 2010), in which the leading edge of the dye was tracked past Station 5. The excursion during the May 24th ebb tide started around 10:45 AM and ended around 5:15 PM. The end of the leading edge was found with the Turner 10-AU fluorometer at 4:23 PM near Lanes Island. The dye was first detected at Station 1 at 11:07 AM and at Station 3 at 12:03 PM, with a travel time of 0.93 hours between Stations 1 and 3. Average WWTP flows during this time were 0.67 MGD. The distance between Stations 1 and 3 was 0.83 miles or 0.72 nautical miles (nm). Therefore, the average velocity was about 0.89 miles/hr (0.83 miles/0.93 hours) or 0.77 knots (0.72 nm/0.93 hours). This velocity was a little higher than the average velocity of 0.57 miles/hr determined by the drogues three days prior on May 21, 2010. The distance between the WWTP diffuser and Station 3 was approximately 1.03 miles, so if the faster/more conservative velocity of 0.89 miles/hr were applied, it would take 1.16 hours or 70 minutes for sewage to reach shellfish at Station 3 (near the mouths of the Royal River and the Cousins River) in the event of a WWTP failure or loss of disinfection. This travel time would decrease under elevated WWTP flow conditions, i.e. effluent flows greater than 0.67 MGD would travel faster to the mouths of the rivers. For example, flows greater than 0.77 MGD (15% higher than 0.67 MGD) could result in effluent reaching the mouths in less than one hour.

3.5 Dye Readings at Cage Stations

Dye readings recorded by the submersible WET Labs units and boat tracking units within a 50 meter buffer area at each of the station locations are shown in Figures 3 - 7. The tidal depth (in feet) is also plotted based on the CTD readings. Any readings at or below background levels (i.e., readings measured by the submersible WET Labs units prior to the dye injection) were removed from the graphs. Steady state concentrations calculated using the superposition method (Kirkpatrick, 1993) and based on the half tidal day low tide, peak 1 hour, and average levels are plotted in Figures 3 - 7.

Figure 3 shows the dye concentration levels at Station 1 over the course of the study. As expected, the peak dye concentration occurred during the low tide following the dye injection period on May 24, 2010. The maximum concentration was 6.1 ppb, which equated to a dilution of 315 for the first tidal cycle. In the Royal River, dye was detected from the top to the bottom of the water column, including near Stations 1 and 2. In general, the depth of the tidal range near Stations (Cages) 1 and 2 was 1 – 12 feet. The highest concentration detected by boat tracking near Station 1 was 47.9 ppb, which is much higher than the maximum concentration of 6.1 ppb detected by the submersible fluorometer at that station. The dye was pretty well distributed at Stations 3, and 4, with similar levels of dye found near the bottom of the water column as at the surface for many measurements. However, for Stations 2 and 5, dye concentrations measured by the submersible fluorometers were high than concentrations detected via boat tracking. Profiles of the water column will be discussed in more depth later in this report.

The dye concentrations for successive tidal cycles were added to ascertain the super position concentrations for Station 1 (Figure 3). For the peak 1 hour concentration levels, the steady state dilution was calculated to be 256. The steady state dilution based on average concentrations was 659. The peak 1 hour and average values for Station 2 were almost identical to those values found at Station 1.

Figures 4 – 7 show the dye concentration levels and steady state dilution values (low tide, peak 1 hour, and average for each half tidal day) for Stations 2 – 5 over the course of the study. The peak 1 hour steady state dilution values represent a more conservative estimate of steady state dilution than the average values, but a more realistic estimate than the maximum values. For Station 2, the peak 1 hour steady state dilution was 249, which does not represent any increase in dilution from Station 1, which actually had a slightly higher dilution of 256. The peak 1 hour steady state dilution for Station 3 was 431, which was an increase in dilution of only 1.5:1 from the dilution achieved at Station 2. Until the dye passed Station 3 and left the mouth of the Royal River, it was fairly evenly distributed throughout the length, width, and depth of the river from the WWTP diffuser to the river's mouth.

After Station 2, the half tidal day peak 1 hour dilution levels followed the pattern of increasing dilution with increasing distance for Stations 2 to 5. Figure 8 shows the relationship of distance vs. dilution. As seen in Figure 8, there was a small increase in dilution between Stations (Cages) 2 to 4 but a very large increase in dilution from Station 4 to Station 5, which were both past the mouth of the Royal River and subject to much more tidal mixing than the other stations. The same relationship of increasing dilution with increasing distance was seen when looking at the half tidal day average values in Figures 4 – 7 as well.

At each of the stations, there is a clear relationship between dye levels and tidal depths, with higher concentrations of dye seen at low tide (with less water to dilute the dye) and lower concentrations of dye seen at high tide (with more water to dilute the dye). This relationship was evident even at the farthest station from the outfall, Station 5. Towards the end of the submersible fluorometer deployment, on June 1, 2010, this relationship continued, with peaks visible at low tide at Stations 2, 4, and 5. The dye signal was strongest at Stations 4 and 5 that day, whereas the dye signal was harder to detect at Stations 1 - 3. This indicates that the dye had moved farther down river by the fourth and final day of the study, but that it could still be detected at low concentrations (< 0.6 ppb) at distances over 2 miles from the outfall.

As stated above and presented in Figures 6 and 7, the dilution for the steady state peak 1 hour concentrations for Station 4 and 5 were 596 and 4347, respectively. Therefore, the 1000:1 steady state dilution line for the Yarmouth WWTP effluent can be estimated to fall between those two stations, but closer to Station 4. Based on Figure 8, the 1000:1 Dilution line for a 0.6 MGD flow can be estimated to occur at approximately 2.37 miles, but this estimate is based solely on the steady state half tidal day peak 1 hour dilution levels for Stations 4 and 5 collected by the submersible fluorometers. Additional considerations are taken into effect later in this report.

3.6 Dye Readings by Tracking Fluorometers on Day 1

One of the advantages of the submersible fluorometers attached to the cage stations was that they could detect dye every ten minutes for thirty second over the entire four day cycle of the study and could therefore pick up dye readings at depth during hours in which boat tracking was not possible. However, while the submersible fluorometers determined the dye levels reaching the oyster cages throughout the course of the study, boat tracking was conducted with two onboard fluorometers (the WET Labs FLRHRT-586 and a Turner 10-AU) to track the dye past the cages and to determine the shape and edges of the dye plume as it traveled down and past the Royal River.

Figures 9 and 10 represent the 5-point moving average concentration values and the corresponding dilution levels for the first day of the study (May 24, 2010) as determined by the WET Labs 586 fluorometer. The raw data used to create these figures (in Excel sheets) can be provided upon request. All 5-point moving average concentrations in the Royal River were less than 50 ppb. Since 1924 ppb of dye were injected, this indicates significant mixing in the near-field mixing zone of the diffuser. The minimum dilution found on May 24th, at 3:25 PM when the ebb tide was near its lowest, was 40:1, equivalent to a 5-point moving average concentration of 47.9 ppb. This level was found near Station 1, less than 0.20 miles from the diffuser. During the 2002 study, the minimum dilution found was less than 19:1 (in the near-field mixing zone). Therefore, the minimum dilution increased by over twice as much from the 2002 study to the 2010 study, most likely due to the installation of the new diffuser.

The minimum dilution found on May 24th with the Turner 10-AU fluorometer was 56:1, right over the diffuser. A Turner 10-AU was also used in the 2002 study, so comparing the Turner results side-by-side, the minimum dilution increased by three times as much from the 2002 study to the 2010 study. As noted in the 2002 final study report, Wright-Pierce estimated that the new diffuser would improve dilution ratios of the effluent from the range of 3:1 – 8:1 to the range of 20:1 – 100:1 based on CORMIX modeling. It appears that the improvement in dilution is on the lower end of the estimated 3:1 – 8:1 range. However, the observed minimum dilution levels of 40:1 – 56:1 in the vicinity of the diffuser are significant and represent a good amount of initial dilution. During the study, dye was also observed in an overland flow along the shoreline that indicated a leak in the buried effluent pipe. EPA worked quickly to address this problem with the WWTP.

Figure 9 shows that significant levels of the dye-tagged effluent (0.5 – 1.0 ppb) traveled up the Cousins River and that the effluent plume traveled directly along the path from Station 3 to Station 4 to Station 5. The dye did not appear to spread out far from the narrow path along which the cage stations were placed, so the location of the stations was ideal for receiving WWTP effluent.

Dye levels of 1.0 – 5.0 ppb were observed just past Station 4. However, as seen in Figure 10 and recorded with the WET Labs 586 fluorometer, dilution levels of 1000:1 and less (concentrations of 2.0 ppb and less) near the water's surface ended about mid-way between Stations 2 and 3. Nevertheless, dye continued to reach Stations 4 and 5 on successive days of the study, as seen in the data collected by the submersible fluorometers, and the steady state 1000:1 dilution was determined to be past Station 4.

3.7 Dye Readings by Surface Tracking Fluorometers on Consecutive Days

Figures 11 - 14 represent the WET Labs FLRHRT-586 fluorometer 5-point moving average concentration values and the corresponding dilution levels for each consecutive day of the study (May 25, 2010 and May 26, 2010). The raw data used to create these figures (in Excel sheets) can be provided upon request.

As can be seen in Figure 11, on the second day of the study (May 25th), higher levels of dye were found northwest of the diffuser and Station 1 (upstream in the Royal River) than were found near Stations 2, 3, 4, and 5. Concentrations between 0.5 – 1.0 ppb were found up to Station 2, but past Station 2, dye concentrations were mostly in the 0.01 – 0.5 ppb range. Some of the highest levels of dye found on the second day were in the area just past the Yarmouth Boat Yard and the bridge but prior to the freshwater input. However, these high levels were found at depths of 5 – 6 feet, not at the surface, and are discussed more in the next section.

Dye levels greater than the limit of detection of the fluorometer instruments (0.01 ppb) but less than 0.5 ppb were detected over half a mile past Station 4 and just prior to Station 5 on May 25, 2010. The distance between the WWTP outfall and the leading edge of the dye was around 2.2 miles. However, as demonstrated in Figure 12, the dilution level at this distance was greater than 10,000 and was in the range of 50,000 – 100,000. Dilution values in the range of 1000 – 5000 were seen upstream of and around Station 2, but not past Station 2 in the direction of the outgoing tide. Figure 12 shows that dilution levels up to 10,000 were seen until the dye reached the mouth of the Royal River, but the dye became better dispersed past the mouth. Figure 12 also shows that dye concentrations between 10,000 – 50,000 ppb were found in the Cousins River on the second day of the study.

Figure 13 shows WET Labs FLRHRT-586 tracking from the diffuser up to and past Station 5 and up the Cousins River on the third day of the study, May 26, 2010. No dye was detected to the south of the cage stations, as shown. The dye concentrations detected via surface tracking on the third day were almost entirely in the range of 0.01 to 0.5 ppb or less than 0.01 ppb. Once again, as had been seen on the first and second days of the study, the dye-tagged effluent became more dispersed after it passed Station 3 and the mouth of the Royal River, with detectable levels heading up into the Cousins River. Dye concentrations up to 0.5 ppb were detected consistently throughout the length of the Cousins. Significant levels of dye at Stations 4 and 5 were not detected via surface tracking on the third day, but low levels of dye (0.01 – 0.5 ppb) were periodically detected between Stations 4 and 5 and even past Station 5 near Little Island.

Figure 14 shows that dilution levels were greater than 10,000 at the surface past Station 2 on the third day, except in the Cousins River, where some calculated dilution levels were less than 10,000. The smallest levels of dilution were observed right near the diffuser and upstream in the Cousins River.

3.8 Profiles of Dye at Depth

The Turner 10-AU onboard fluorometer was used to conduct profiles of the dye at different depths in order to determine the vertical distribution of dye in the water column. Figures 15 and

16 show dye profiles recorded with the Turner 10-AU for the first two days of the study. Depth was measured with a marked rope and recorded alongside the dye concentration readings in a notebook.

As seen in Figure 15, near the diffuser and Station 1, as well as farther upstream in the Royal River, higher levels of dye were found at lower depths on the first day of the study. For example, at depths of 5 feet near Station 1, the dye concentrations found were up to 1.4 ppb, whereas at depths of 0 – 1.5 feet, the dye concentrations found were in the range of 0.5 – 0.7 ppb – about half as much dye. Near the diffuser, dye concentrations up to 3 ppb were found at 8.5 feet of depth – almost six times as high as the 0.4 - 0.6 ppb levels found near the surface. Since the diffuser was submerged, the dye tended to stay submerged as well. It gradually rose to the surface as it traveled downstream. By the time the dye-tagged effluent reached Station 3, higher levels of dye were being found at the surface than at lower depths.

Figure 16 shows dye concentrations for depth profiles conducted on the second day of the study, as recorded by the Turner 10-AU. On this day, higher levels of dye were found near the surface than at depth in some cases. For example, a profile taken at the boat dock (west/upstream of the diffuser) recorded dye levels of 0.6 – 0.9 ppb from the surface down to 2.5 feet deep, but at depths of 6 feet and lower, dye levels were less than 0.1 ppb. This same trend was observed at Station 2 (see Figure 16). However, in other cases, higher levels of dye were found at lower depths than at the surface particularly near the bridge past the Yarmouth Boat Yard. Only background levels were detected at the surface, but at six feet deep, dye concentrations greater than 1 ppb were recorded. These were the highest dye concentrations found with the Turner 10-AU on day two of the study. There is a large freshwater input into the Royal River in this area, and it appears that a saltwater wedge was created that pushed the dye-tagged effluent to a depth of six feet and trapped it there in the day following the dye injection. As time passed, the trapped dye eventually traveled down the Royal River while rising to the surface and dispersing, particularly once it reached the mouth of the river.

Profiles were conducted on the third day of the study as well, but for many of the profiles, only background levels were detected. For the other profiles, the dye concentrations found at depth were the same as those found at the surface and were very low. Therefore, the profiles for the third day are not depicted in an ArcGIS figure.

The tracking and profile fluorometer data matches fairly well with the data collected by the submersible fluorometers, though the submersibles picked up slightly higher levels of dye because they were operating throughout the entire study, whereas the boat tracking and profiles were only conducted during daylight hours in which the boats could be operated. This was particularly true on the third day of the study when the dye-tagged effluent was well dispersed.

3.9 Projections for Different Wastewater Treatment Plant Flows

This study, which was conducted in May 2010, was during a relatively low wastewater treatment plant flow period due to warm weather and a low amount of storm water, even though there had been significant rainfall levels in the week before the study. With the low flow values, the calculated dilution values will be greater than would occur with higher flows. The average

WWTP flow during the 12.4 hour dye injection period on May 24, 2010 was 0.60 MGD based on flow data provided by the Yarmouth WWTP. The maximum flow rate during the dye injection period was 1.316 MGD. Thus, the ratio of maximum flow to average flow during the comprehensive dye injection study was 2.19.

Based on the results of this study, FDA developed a model to predict how dilution of the WWTP effluent in the Royal River would change at each of the station locations based on higher WWTP flows and lower WWTP flows than those recorded during the study. The model also predicts how the estimated MSC and NoV GI and GII levels will change in the estuary with changing levels in the influent and effluent. This model, the "Royal River Dilution Model", was sent to the DMR and other study partners on April 13, 2011, with a revised version sent the following day. A person using the model can enter a new flow value into the model and immediately see how the dilution levels in the estuary will change. For example, the steady state peak 1 hour dilution levels for Stations 1, 2, 3, 4, and 5 during the study were 256, 249, 431, 596, and 4347, respectively (see Figures 3-7). These were based on a WWTP flow rate of 0.6 MGD. When the WWTP flow rate is increased to 1.50 MGD in the model, which is more representative of high-end flows at the WWTP, the steady state peak 1 hour dilution levels at the station locations are reduced to 102, 100, 172, 238, and 1739. This represents an overall 2.5-fold lower dilution of WWTP effluent.

FDA also modeled how dilution in the Cousins River would change under different flow conditions. Figure 12 shows the accumulated surface concentration and associated dilution for the May 24 – 26 study period based on boat tracking of the dye. As can be seen in the figure, surface concentrations detected in the Cousins River were primarily in the 0.4 – 0.6 ppb range. However, Figure 13 shows what predicted surface concentration levels would be based on an elevated WWTP flow rate of 2.5 MGD. This is the flow level at which the WWTP's aeration is reduced or shut down (personal communication with WWTP operator Tom Connolly). These results were modeled in ArcGIS 10.0 by scaling up the WWTP flows and the associated dye concentration levels in the final effluent. As can be seen in this figure, surface concentration levels mainly in the range of 1 – 5 ppb would be expected in the Cousins River under this scenario. This would equate to dilutions of 126:1 to 630:1.

Figure 14 shows daily precipitation and instantaneous flow rates at the Yarmouth WWTP for the month of December 2007 This figure also shows MSC results in shellfish from the Cousins River collected on 12/27/07 and on two other days. Although WWTP flows were below 2.5 MGD during this period and below the 1.00 inch rainfall trigger, the shellfish samples collected on 12/27/07 contained high levels of MSC in the range of 4381 – 7361 PFU/100g. This result could be due in part to an exceedance of the WWTP's monthly average design flow (1.31 MGD) on several occasions in the weeks prior to the sampling date.

FDA conducted a follow-up study in 2012, as discussed later in this report, the results of which were reported in "Supplemental Report to the 2010 Hydrographic Study in Yarmouth, Maine – Shoreline Survey Source Identification". As noted in that supplemental report, FDA determined that WWTP flows can have a significant impact on MSC levels detected in the final effluent. Therefore, it's important to consider projections of decreased dilution for higher WWTP flows for both the Royal and Cousins Rivers when determining how to manage the growing areas.

3.10 Microbiological Analysis of WWTP Influent and Effluent

The microbial indicator results for the Yarmouth WWTP influent and effluent on April 7, 2010 – six weeks prior to the study – are presented in Figure 17a. As shown in the figure, the FC and EC levels in the influent wastewater were at concentrations of 7.0×10^5 /100 ml. Levels of FC and EC were found to be low in the effluent at concentrations of <1/ 100 ml. Furthermore MSC levels present in the effluent were at densities below the level of detection (10 pfu/ 100 ml)

The indicator microorganism results for the Yarmouth WWTP influent and effluent during the hydrographic dye study (May 24 – 26, 2010) are shown in Figure 17b. The average FC level detected in the influent was 7.0×10^6 FC/100 ml – five times the 1.4×10^6 FC MPN/100 ml literature values. FC and EC results in the pre-chlorinated effluent were as high as 28,000 FC/100 ml and 25,000 EC/100 ml, respectively.

MSC levels detected in the influent and pre-chlorinated effluent were as high as 528,000 PFU/100 ml and 1200 PFU/100 ml, respectively. MSC results in the final effluent did not indicate the likely presence of viruses under the low WWTP flow levels (0.6 MGD) that occurred during the study (<9.9 PFU/100ml).

3.11 Microbiological Analysis of Oysters at Cage Stations

Figure 18 shows the results of FDA microbiological testing of the oyster sentinels that was conducted after the oysters were recovered and shipped to the GCSL on June 2, 2010. Higher levels of FC, EC, and MSC were found in the oysters than in the WWTP effluent, with an average 300-fold higher FC/100 g detection level at the stations closest to the diffuser (Stations 1, 2, and 3). FC and EC levels decreased with increasing distance from the diffuser, as expected, but MSC levels did not follow this trend. As noted in Figure 18, NoV GI, NoV GII, and Adenovirus were "non-detected" in oysters from all five of the stations. This finding is consistent with the very low microbial indicator findings in the Yarmouth WWTP effluent, including the lack of MSC detection.

3.12 Microbiological Analysis of Soft-Shell Clams Collected Near Cage Stations

Figures 21 – 24 contain Spinney Creek microbial indicator results (FC and/or MSC) for soft-shell clams harvested near Stations 1, 2, and 3 in the Royal River during the study. NoV GI and GII results, determined by the FDA's GCSL, are also provided in Figure 19.

Figure 19 shows the results for sampling performed on May 25, 2010. The FC results for Stations 1, 2, and 3 were 93, 130, and 110 FC/100 g, respectively. These results are similar to the FC findings in oyster sentinels a week later on June 2, 2010 – 170, 110, and 170 FC/100 g, respectively (see Figure 18).

Figure 19 also shows MSC results in soft-shell clams for two trials conducted during the study. The testing was performed in triplicate. The average MSC levels at Stations 1, 2, and 3 during the first trial were 212, 221, and 1024 PFU/100g, respectively. During the second trial, samples

were also taken from a location between Stations 2 and 3 (named "Station 2.5" in Figure 19) and from Station 4. The average MSC levels at Stations 1, 2, 2.5, and 4 were 111, 56, 190, and 195 PFU/100g, respectively. MSC levels did not decrease with increasing distance from the WWTP, but as was observed during the dye study, the dye-tagged effluent was fairly evenly distributed throughout the Royal River from Station 1 to the river mouth. Even past the river mouth at Station 4, levels of dye in the range of 1.0 – 5.0 ppb were still found on the first day of the study (see Figure 9).

In all cases, during both the first and second trial, the average MSC findings at Stations 1, 2, 2.5, 3, and 4 were above 50 PFU/100g and were considered significant.

Figure 20 shows triplicate MSC findings in soft-shell clams collected near Stations 1, 2, and 3 on May 24th with standard error bars. From this figure it's easy to see the similarity in the MSC results at Stations 1 and 2, where dye was evenly distributed, as opposed to an increase in MSC at Station 3. One possible explanation for the higher MSC results at Station 3 was that another human pollution source was contributing to the MSC impact at that station, perhaps from the Cousins River. FDA conducted a follow-up study to investigate the possibility of a separate shoreline pollution source in 2012, and the results are described in the supplemental report "Hydrographic Study of Yarmouth Maine Waste Water Treatment Plant Effluent, Report of Findings from the August 17 – August 22, 2002 Study Period". As noted in that report, FDA did not locate any other significant MSC contributing pollution sources in the area.

Method and Biological Variation are shown in Figure 21. The variation for Station 3 is mid-way between the variation observed at Stations 1 and 2 on May 24th, so variation does not appear to be a significant factor in the MSC increase noted at Station 3.

While MSC levels were non-detectable in the oyster sentinels, MSC were found in the natural set soft-shell clams tested during the study. On May 19 and 20, 2010, about 4 - 5 days prior to the study, about 1 – 2 inches of rain fell in the Yarmouth area, according to NOAA's National Weather Service (http://water.weather.gov/precip/). As demonstrated by the hydrographic dye study, it took about 4 – 5 days for dye-tagged effluent to be flushed from the river near Stations 1, 2, and 3 (see Figures 3 – 5), which is where the soft-shell clams were harvested. NOAA does not report rainfall occurring during the timeframe of the study itself and rainfall was not observed by FDA during the study.

Since the oyster cages were placed in the water 4 - 5 days after the rainfall occurred, whereas the natural set soft-shell clams were in the water during the rainfall event, the clams would have been more exposed to increased WWTP flows and higher MSC exposure due to the rainfall on May 19 and 20, 2010. Furthermore, the clams were exposed to WWTP effluent build-up in the Royal River that occurred over their lifetimes, while the oyster sentinels were only exposed to effluent for periods of approximately two weeks. The oysters were also depurated prior to deployment. The clams were not depurated at any point prior to testing, as they were harvested from their natural environment.

3.13 Short Term Failure - Dilution and Anticipated Fecal Coliform (FC) Concentrations at One Ebb

Dilution of the dye-tagged effluent can be related to all of the low tide dye readings taken from May 24 – June 1, 2010 using the submersible fluorometers. Dilution is physical and is computed by dividing the dye concentration found at locations in the estuary into the dye concentration added to the WWTP effluent. This is the principle on which the "Royal River Dilution Model" is based. Therefore, the low tide dye readings found on May 24, 2010, the first day of the dye feed, and the FC counts found in the influent on that day can be used to estimate the FC counts that would occur at the 1.5, 1.0, and 0.5 ppb contours in a short-term WWTP failure scenario.

Table 1. The following table provides the dilution values for 0.5, 1.0, and 1.5 ppb concentrations. Also shown are anticipated fecal coliform (FC) concentrations, if a short term lapse in disinfection should occur (single ebb tide and assuming no decay). Note that the 0.5 ppb level was found past Station 5 on the first day of the study (see Figure 9). A typical literature based value of 1.4×10^6 FC MPN/100 ml as the anticipated fecal coliform count for raw, untreated wastewater was used to represent the worst-case, total failure scenario. However, actual influent data for the Yarmouth WWTP is presented in Figures 17a and 17b and was also used in the analysis. FDA testing during the dye study period (Figure 17b) found a FC level of 7.0×10^6 FC MPN/100 ml in the Yarmouth WWTP influent (five times the literature value). This value may be more reflective of the average FC levels found at the Yarmouth WWTP and is also used in Table 1.

Table 1: Dilution and Anticipated Fecal Coliform Concentration on Ebb Tide

Dye Contour (ppb)	Dilution with Respect to FC with no decay	Anticipated Concentration (FC/100 ml)	Anticipated Concentration (FC/100 ml)
		With 1.4×10^6 FC/100 ml	With 7.0×10^6 FC/100 ml
1.5	1283:1	1091	5456
1.0	1924:1	728	3638
0.5	3848:1	364	1819
0.1	19240:1	73	364

A short-term raw sewage failure could result in deteriorated water quality in a single ebb tide (Table 1). For low WWTP flow periods (considering the single day ebb tide results), a failure could result in fecal coliform densities ranging from 73 – 364 FC/100 ml at the 0.1 ppb contour, which during the study was around 4.0 miles from the WWTP diffuser (just past Station 5) as seen in Figure 12. For high flow periods, which are more likely to result in raw sewage failures, the fecal coliform densities would be expected to be even higher and the velocity at which the sewage travels in the growing areas would be higher as well.

Under Scenario 1 for sizing prohibited areas (see Section 1.2 of this report), in consideration of effluent discharged from a WWTP under failure conditions, a prohibitive zone must provide a sufficient amount of dilution to dilute the effluent discharged under failure conditions to the FC standard of 14 MPN/100 ml within the prohibitive zone. Therefore, the prohibited zone would

need to extend past 4.0 miles from the WWTP outfall in order to achieve the 14 MPN/100 ml standard under this classification scenario.

If Scenario 2 is considered, then the conditional growing area would need to extend from the 1000:1 dilution line to a distance greater than 4.0 miles from the WWTP so that the 14 MPN standard could be met within the conditional area zone before the effluent reaches an approved area. As can be seen in Figure 9, on the day of the dye injection, the dye went from levels of 0.01 – 0.5 ppb down to non-detectable levels between Blaney Point on Cousins Island and the southern tip of Little Moshier Island. This is where a conditionally approved growing area could transition to an approved area under Scenario 2.

3.14 Determination of a 1000:1

Under Scenario 2 for sizing prohibited areas (see Section 1.2), the size of the prohibited zone can be reduced and a conditional area can be established if a 1000:1 dilution zone is achieved and other conditions are met.

The 1000:1 dilution line changes throughout the course of the dye study, so the steady state condition of the estuary should be assessed to estimate where the 1000:1 dilution line will be when the rate of effluent entering the system from the WWTP outfall is the same as the rate of effluent being pushed out be the tides. To do this, we need to rely on the data collected from the submersible fluorometers attached to the station cages, since this data was being recorded on a continuous basis throughout the study. As noted in Section 3.6, the 1000:1 dilution line based on the peak 1 hour superposition concentrations calculated for Stations 4 and 5 was estimated to occur after Station 4 at around 2.37 miles away from the WWTP outfall.

The maximum dye concentration reading by the submersible fluorometer at Station 4 was 1.83 ppb (see Figure 6) and at Station 5 was 0.57 ppb (see Figure 7) for the first day of the study. Figure 9 shows that the dye levels detected via boat tracking at the water's surface on the same day near Station 4 ranged from 0.5 – 1.0 ppb (with a few readings over 1.0 ppb) and near Station 5 ranged from 0 – 0.5 ppb. These levels are very consistent with those detected by the submersible fluorometers. While the dye-tagged effluent was less evenly distributed near the diffuser and Station 1, once it reached Stations 4 and 5, the plume appeared to be fairly evenly distributed throughout the water column. On the second and third days of the study, the submersible fluorometers picked up more dye than the boat tracking fluorometers and provided the more conservative estimates of dilution. Therefore, the dilution estimates made using the submersible fluorometer data at Stations 4 and 5 on the bottom of the ocean floor do not need to be adjusted to account for higher concentration readings at the surface

Scenario 2 for sizing prohibited areas (see Section 1.2) also states that if a conditional shellfish growing area is established based on the operation of the Yarmouth WWTP, it will need to account for not only a minimum of a 1000:1 dilution, but also allow for an adequate notification time to properly manage the area. As previously noted in Section 3.4, the travel time of the dye was approximately 0.57 – 0.89 miles/hr. Since the 1000:1 dilution line falls around 2.37 miles from the outfall, the notification time would need to be in the range of 2.7 – 4.2 hours or less since travel time can decrease when flows are elevated, which is typical of failure conditions.

3.15 Prohibited Area Associated with Conditionally Approved Classification

As previously indicated in Section 1.2, in order to maximize the size of the shellfish growing area and reduce the size of the prohibited zone, a conditionally approved zone may be managed if three factors are met: 1) adequate dilution of effluent within the prohibitive area in consideration of the WWTP operating under normal conditions (i.e., 1000:1 dilution); 2) sufficient amount of time to close conditional area to harvesting in the event of a failure (a management plan is required); and 3) adequate amount of dilution within the conditional area to dilute effluent discharged under failure conditions (i.e., meeting a fecal coliform standard of 14 MPN/100 ml).

A conditionally approved area management plan would include performance standards for the wastewater treatment plant, adequate monitoring and alarms, and a management plan that insures that harvest might be done, but only when the WWTP, disinfection systems, recording devices and alarms are in operation. Added effluent travel time in long outfalls can be considered in the travel time for the estimated arrival of effluent. Notification time is usually the controlling factor in performance standards and management plans. In some situations, alarm and notification procedures for shellfish control officials, the industry doing harvesting, and harvesting and growing area control officials are so effective that notification times can be reduced to a few hours or less. In these situations, the notification times may be so short that adequate dilution does not occur for normal secondary treated effluent in order to mitigate virus impacts. If these situations should occur, a minimum effluent dilution of 1000:1 is still advised. Even if the notification time between WWTP breakdown and the ceasing of harvest is less than the travel time to obtain the minimum dilution, the location of minimum dilution would be the controlling factor for setting the boundary of the conditionally approved growing area.

4.0 Spinney Creek Pilot Study

Spinney Creek Shellfish, Inc. (Spinney Creek) assisted with this study and provided some of the MSC figures, as previously noted. FDA met with Spinney Creek on March 17, 2011 to discuss a proposal to permit harvesting of soft-shell clams in the Royal River in the proximity of Station 2 (see Figure 1). The proposal allows harvesting during the summer season, when the viral risk is lower due to higher temperatures, if Spinney Creek performs MSC testing on harvested clams to demonstrate that MSC levels are less than 50 PFU/100 grams and if the clams are depurated for a minimum of two days. Spinney Creek presented seasonal MSC data from the Royal River and another river in Maine, the Fore River, to support the proposal. This data is presented in Figure 22 in this report.

FDA agreed to assist in the pilot study for this proposal. However, FDA stated that harvesting should not take place near Station 2, but rather only at locations past Station 2.5 (located between Stations 2 and 3) since the available MSC and NoV results only supported a pilot for seasonal harvesting at Station 2.5. Samples of shellfish harvested during this pilot study were analyzed by FDA's Gulf Coast Seafood Laboratory (GCSL) in Dauphin Island, AL for NoV GI and GII. MSC analysis was performed by Spinney Creek Shellfish.

FDA also proposed testing the Yarmouth WWTP's influent and effluent as part of the pilot study, since the WWTP's microbial indicator levels were very low during the hydrographic dye study. It was unclear at that time whether the levels at the WWTP were typically very low due to the efficiency of the WWTP and high retention times or if the levels were low due to dry weather conditions and low WWTP flow levels at the time of the study. If the WWTP's influent and effluent microbial indicator levels were found to be consistently lower than those average levels seen in FDA studies and in the literature, this is a factor that could be considered in sizing a conditional growing area and as part of the pilot study analysis.

The pilot study is still ongoing. Results thus far indicate that temperature and seasonality are important factors in the presence of MSC in soft-shell clams in the Royal River, with MSC levels decreasing significantly under warmer temperatures. For this reason, certain seasonal exemptions could be applicable for growing area management of the Royal River.

5.0 Conclusions of Hydrographic Dye Study Conducted in May 2010

The weather was unexpectedly dry and warm (around 90°F) during the study, which may have influenced the low flow level at the WWTP (0.6 MGD) and the resulting low or non-detect levels of microbial indicators and viruses in the final effluent and the oyster sentinels. However, significant levels of MSC were found in the natural set soft-shell clams collected near the sentinel stations. While oysters were depurated prior to deployment and only exposed to WWTP effluent for less than two weeks during low flow, dry weather conditions, the soft-shell clams were exposed to a significant rainfall event and higher WWTP flows the week prior and had more time to accumulate MSC and viruses. The findings in the soft-shell clams are likely more indicative of higher WWTP flow conditions and should therefore be considered in growing area management recommendations. While the microbial testing results in the oysters did not indicate a concern during the study, these results only represented a brief snapshot in time. Therefore, during the ongoing Spinney Creek pilot study, continued testing of soft-shell clams for NoV GI and GII and viral indicators, like MSC, is recommended over several months to provide a better determination of viral impacts from the WWTP on shellfish in the Royal River.

Based on a steady state dilution analysis of the data collected by the submersible fluorometers at each of the sentinel stations, the 1000:1 steady state peak 1 hour dilution line was estimated to occur near Station 4, about 2.37 miles from the WWTP diffuser. Based on the FC levels found in the WWTP influent and the dilution findings in the estuary, in the event of a WWTP failure, the effluent would need to travel a minimum of 2.5 miles to meet the 14 MPN/100g FC standard for "approved" areas. This standard can be achieved by establishing a conditional growing area boundary line between Blaney Point on Cousins Island and the southern tip of Little Moshier Island. Therefore, under Scenario 2 as described in Section 1.2 of this report, FDA recommends establishing a conditionally approved growing area zone from the 1000:1 dilution line to the Blaney Point boundary line as shown in light red in Figure 23, if sufficient notification time exists to close the area in the event of a WWTP failure. [A more specific ArcGIS map of Figure 23 can be created upon request].

The distance from the WWTP diffuser to the 1000:1 line is 2.37 miles, and as previously noted in this report, it would take at least 1.7 hours for effluent to reach the 1000:1 line in the event of

a WWTP failure. Therefore, FDA recommends that the permitted notification time for a failure event be 1.7 hours or less in managing the proposed conditional growing area for non-depurated product, if applicable. This condition would not apply to product intended for depuration, since the 2 – 3 day depuration time would allow more than enough time to notify processors of a WWTP failure and to prevent contaminated shellfish from reaching the market.

Significant levels of dye reached the Cousins River during the study, including levels up to 1 ppb on the first day of the study, and were found throughout the river. Dye levels were particularly high when water levels in the Cousins were very low at the end of the ebb tide. Dye concentrations were similar to those found near Stations 3 and 4. Although there were no submersible fluorometers positioned in the Cousins River during the study that would allow a steady state dilution analysis, small levels of dye were found in the Cousins on the second and third day of the study (see Figures 11 and 13), indicating WWTP effluent build-up is also occurring there. In addition, levels of MSC detected in shellfish from the Cousins River and in the Royal River were significantly elevated levels during winter months (see Figures 14 and 22).

During the study, WWTP flows were low and retention times were high. There was good removal of FC, *E. coli*, and MSC from the influent to the final effluent. FDA recommends continued testing of the WWTP's influent and effluent for viruses (not just indicators) to determine if WWTP efficiency is particularly high relative to other WWTPs and what patterns emerge in regards to MSC and virus detection under higher flows.

There were no viruses found in the oyster sentinels during the short time frame of the study, but it is difficult to time a study to coincide with high virus input. Testing natural set soft-shell clams as part of the pilot study will over a long period of time provide more opportunities to study the true viral inputs into the Royal River system. The soft-shell clams were more susceptible to MSC during the study than the sentinel oysters and should make good test subjects. FDA's GCSL will conduct testing with clams sent by Spinney Creek during the proposed pilot study. Once more information is available regarding the WWTP's influent and effluent and NoV GI and GII levels in soft-shell clams in the Royal River, FDA can provide further recommendations regarding Spinney Creek's proposal for harvesting within the Royal River and outside the recommended conditionally approved growing area.

6.0 Follow-Up Study Conducted in April 2012

FDA conducted a follow-up investigation (shoreline survey) from April 23 – 26, 2012 to identify actual or potential pollution sources beyond the Yarmouth WWTP that could be contributing to the MSC findings in the soft-shell clams. FDA also used this study period to determine how the Yarmouth WWTP performed under wet weather flow conditions by assessing the plant's influent and effluent and soft-shell clams in the Royal River for FC, *E. coli*, and MSC after a large (3.99 in) rainfall event. The results of these assessments are presented in the document "Supplemental Report to the 2010 Hydrographic Study in Yarmouth, Maine – Shoreline Survey Source Identification".

The supplemental report concludes that the Yarmouth WWTP can remove FC and *E. coli* under high flow conditions but that it does not perform well at removing MSC under high flows. After

the large rainfall event, instantaneous flows at the WWTP were as high as 4.095 MGD and MSC levels were as high as 38,200 MSC/100 ml in the WWTP's effluent. The elevated MSC levels only stabilized when flows returned to 1.0 MGD – below the plant's permitted discharge level of 1.31 MGD.

FDA looked at a number of other pollution sources in the vicinity of the Royal and Cousins Rivers but could not locate any other source with high enough flows and MSC levels to account for the MSC findings in soft-shell clams in the growing areas. All lift stations were observed to be operating within hydraulic capacities with no apparent overflows during the storm event. Therefore, the supplemental report concludes that the Yarmouth WWTP under high flow conditions is the contributing source of MSC and viruses to the soft-shell clams.

Based on this determination, FDA created a regression curve of MSC in the effluent vs. the WWTP's instantaneous flow rate in order to estimate the levels of MSC expected in the effluent under different flow conditions (Figure 24). For example, Figure 24 shows for a flow level of 1.31 MGD (the WWTP's permitted discharge level), MSC levels in the effluent would be estimated at 166 PFU/100 ml, whereas for a flow level of 2.5 MGD, MSC levels in the effluent would be estimated at 10,636 PFU/100 ml.

7.0 Recommendations Based on All Available Information

After the May 2010 hydrographic dye study and microbiological assessments, the April 2012 follow-up investigation, the Spinney Creek pilot study, and other work conducted by DMR, FDA, and Spinney Creek, FDA currently has access to more data pertaining to the Royal and Cousins Rivers than to any other shellfish growing areas in the United States. When considered collectively, this data supports the following conclusions:

- The Yarmouth WWTP is very efficient at removing FC and *E. coli* indicator bacteria under all types of flow conditions and in meeting its permitted requirements for FC.
- The Yarmouth WWTP is efficient at removing MSC under low flow conditions (i.e., less than the plant's permitted discharge level of 1.31 MGD), but high levels of MSC are detectable in the effluent when wet weather flows are high. For this reason, the WWTP's ability to remove viruses under flows greater than the permitted discharge level of 1.31 MGD is a concern and should be further investigated.
- MSC and NoV have been detected in natural set soft-shell clams harvested from the Royal River and the Cousins River. MSC was detected in soft-shell clams in both the May 2010 study and the April 2012 study, as well as in other analyses conducted by FDA and Spinney Creek.
- The MSC and NoV detected in soft-shell clams in the growing areas appear to be coming from the Yarmouth WWTP under high flow conditions. FDA was not able to locate any other pollution source that could be causing the virus findings in the clams.
- MSC findings in the clams are seasonal/temperature dependent and decrease significantly in the summer months (see Figure 22).
- 1000:1 steady state dilution is achieved approximately 2.37 miles from the WWTP diffuser under typical flow conditions.

- Dye-tagged effluent remained detectable in the growing areas for at least 5 days and a "build-up" of effluent was observed in both the Royal and Cousins Rivers.
- Estimated travel time of the effluent is approximately 0.57 to 0.89 miles/hour. For a conditionally approved area established at 2.37 miles from the WWTP, the response time needed would be 2.7 hours. For conditionally approved areas established even closer to the WWTP diffuser, the response time needed would be less than 2.7 hours.

Based on these findings, FDA recommends that the Royal River and Cousins River be classified as prohibited or conditionally restricted with seasonal management and harvesting limited to the summer months when the viral risk is lowest. FDA recommends that a conditionally approved zone be established 2.37 miles from the WWTP diffuser and extending to the boundary line between Blaney Point on Cousins Island and the southern tip of Little Moshier Island as shown in Figure 23, if DMR can respond to a WWTP failure in less than 2.7 hours. Waters south of the boundary line or outside the red shaded area in Figure 23 could be classified as approved. Given the volume of information available for these growing areas, alternative classification and management plans could be considered if the mitigation of viral and other risks is supported by the data.

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