Appendix 1. Description of Research Method for Analysis of Hormones in Water

The method is part of a (ultra performance) liquid chromatography tandem mass spectrometry (LC/MS/MS) research method (analysis code LCHM) developed at the Organic Geochemistry Research Laboratory to measure hormones, hormone conjugates, steroidal compounds, and phytoestrogens. The following describes sample handling and analysis techniques associated with this method, as followed for the study of water-quality characteristics indicative of wastewater in selected streams in the upper Neuse River Basin, Durham and Orange Counties, North Carolina, from 2004 to 2013.

Upon receipt, samples were defrosted and 1 milliliter (mL) aliquots were pipetted into silanized glass chromatography vials. The remaining sample and sample aliquots were stored at 10 ^{o}C until analysis. Sample aliquots were analyzed for five hormones—17-β-estradiol, 17-α-estradiol, estriol, estrone, and ethynylestradiol—using a Waters (Milford, Mass.) H-Class Bio ultrapressure liquid chromatograph (UPLC)/AB/Sciex (Ottowa, Canada) 5500 tandem mass spectrometer.

A seven-point standard curve was prepared from serial dilutions of a 1 nanogram per microliter (ng/µL) analyte standard mix at concentrations of 0.001, 0.01, 0.05, 0.10, 0.50, 1.0, and 2.0 microgram(s) per liter (µg/L). These standard solutions were also amended with 2 µg/L concentrations of the surrogate standard mix that included the following deuterated compounds: 17-β-estradiol-d_4, estrone-d_4, estriol-d_3, and ethynylestradiol-d_4. In addition, two solutions were prepared: one referred to as A, which contained a 36 µg/L concentration of the surrogate compounds, and a second, referred to as SA, which contained a 36 µg/L concentration of the analyte and surrogate standard mix. Standard curve solutions, environmental samples, and the A and SA solutions were placed in the ultra performance liquid chromatograph (UPLC) refrigerated (4 ^{o}C) autosampler just prior to the start of the analytical run.

The standard curve solutions were analyzed at the beginning of the analytical run using 90 µL injections. Two stacked injections were used to analyze the environmental samples. The sample was first analyzed by drawing 90 µL of sample and 5 µL of the A solution (surrogate mix) and then reanalyzed by drawing 90 µL of sample 5 µL of the SA solution (surrogate and analyte mix). Addition of the 5 µL of the A and SA solutions was equivalent to the mass of each analyte at 2 µg/L in a 90 µL sample. Thus, the concentration of the surrogate standard mix matched the concentration of the surrogate standard mix in the standard curve solutions. Blank solutions of 50:50::methanol:water were analyzed prior to the analysis of each sample and between each A and SA sample pair.

Chromatographic separation was achieved on a 50 x 2.1 millimeter (mm) bridged-ethyl-siloxane/silica hybrid (BEH) column with 1.7 micrometer (µm) packing employing a 5.5-minute gradient separation. Mobile phases A and B consisted of water and methanol, respectively, pumped at a rate of 0.5 milliliters per minute with the column heater set at 40 ^{o}C. The gradient was held at 10 percent B for 0.2 minutes then ramped to 35 percent B at 2 minutes, to 98 percent B at 4 to 4.25 minutes. The column then was equilibrated for 1 minute at 10 percent B prior to the next injection. Concentrated ammonium hydroxide was introduced into the mobile phase (post column) at a rate of 1 µL per minute just prior to entering the MS/MS to enhance the ionization of the hormones. The separated compounds were analyzed using electrospray ionization (ESI) in negative-ion (NI) mode. The MS/MS conditions were as follows: ion spray

voltage, -4,000 volts (V); source temperature, 650 ^{o}C; declustering potential (DP), -60; entrance potential (EP), -13; Gas1, 40; Gas2, 70; curtain gas, 20; collision gas, 7. The collision energy and collision exit potential were optimized for each compound.

All analytes and surrogate standards were analyzed using multiple-reaction monitoring. Two daughter ions were measured from the fragmented deprotonated molecule for each analyte. Criteria for positive identification required that the retention time of the analyte was within 2 seconds of its surrogate equivalent and the ratio of the quantitation to confirming daughter ions for each compound was +/- 25 percent of the average ratio in the standards. Compound concentrations were determined from a linear standard curve with 1/X weighting based on the quantification daughter-ion ratio of the analyte-to-surrogate standard. For 17-α-estradiol, the 17-β-estradiol-d_4 surrogate standard was used. The SA sample analysis for each sample pair was used to assess for matrix effects.