D.1. Introduction
This appendix for the Withers basin Watershed Assessment Report (WAR) provides a brief description of the results obtained from measurement of three types of chemical tracers and one toxicity bioassay. These results were used in concert with FIB concentrations and three types of genotypic assays to support a weight-of-evidence approach for identifying fecal sources in the Withers basin. The results from the bacteria concentration measurements and genotypic source assays are presented in Section 3.4 of the Watershed Assessment Report. This appendix also includes the summary matrix used to collate the chemical tracer and bioassays results into a format for use in Section 3.5.1 of the WAR. This enabled a weight-of-evidence approach in identifying which sites had consistent evidence for bacterial contamination from humans and canines.

The first category of chemical tracers reflects the presence of fecal inputs but these tracers are non-specific for host animal sources. These include turbidity, ammonia, total suspended solids (TSS), volatile suspended solids (VSS), and 5-day biochemical oxygen demand (BOD5). High values are consistent with findings of high FIB concentrations because fecal matter imparts these chemicals to the water. Other sources of these chemicals can be present, so these tracers are best used in a weight-of-evidence context.

The second category of chemical tracers is specific for human inputs. These include optical brighteners and caffeine. Optical brighteners are additives included in laundry detergents and hence are a common component of septic effluents. Their presence can be detected at very low concentrations using fluorometric methods. Optical brighteners do not have natural sources but naturally occurring fluorescent dissolved organic matter can produce false positive results. Optical brighteners degrade rapidly in the presence of sunlight unlike the natural organic matter. This difference in behavior is used to correct optical brightener measurements for interferences from natural organic matter (Cao et al.)
This also suggests that waters with high concentrations of optical brighteners have experienced recent inputs of septic effluents. Caffeine is a widespread component of beverages and some foods that passes largely unmetabolized into human wastes. Sauvé et al. 2012 has defined threshold concentrations that are predictive for co-occurrence of significant fecal bacteria contamination.

The third category of chemical tracers helps define where waters in the drainage system originate and their pathway through the watershed. They are not necessarily specific for fecal inputs. The tracers used in this study that fall in this category include: specific conductivity, temperature, pH, and dissolved oxygen. Since some are elevated by the input of wastewaters, they are recommended for use by stormwater managers as tracers of illicit discharges, most notably specific conductivity (Pitt 2004).

Additional parameters measured as part of the water quality investigation included a bioassay based water fleas (Daphnia magna). This standard bioassay screens for toxic chemicals such as metals, pesticides, and herbicides. This bioassay was used to begin development of a background data set that will inform future efforts at addressing water quality concerns beyond fecal bacteria in the Withers basin.

The details of the sampling design and field work for the Withers basin water quality investigation is provided in Section 3.3. This includes an explanation of the techniques used in visualizing and analyzing the data to realize the weight-of-evidence approach. Graphs and maps for each of the parameters measured are provided in Appendix E. A list of the relevant analytical methods and regulatory water quality standards for the chemical tracers is provided in Table 3.3-3. Acronyms are defined in the front matter of the WAR.

D.2. Results

D.2.1. Non-specific chemical tracers of fecal inputs

Concentrations of several of these tracers exceeded regulatory and informal water quality standards. Most wet (37 of 42; or 88.1%) and dry (23 of 24; or 95.8%) weather samples had five-day biochemical oxygen demand (BOD₅) exceeding the informal water quality standard of 2.0 mg/L (Figure D.2.1-1). High concentrations of total suspended solids (TSS), volatile suspended solids (VSS), and turbidity were also observed. Almost one third of the samples (19 of 66; or 28.8%) had turbidities that contravened the SC DHEC water quality standard of 50 NTU for Class FW waters, particularly during wet weather events (Figure D.2.1-2). Ammonia concentrations were below levels toxic to aquatic organisms (EPA 2009). Concentrations were higher under wet as compared to dry conditions.
**Figure D.2.1-1.** BOD5 concentrations (mg DO/L) in Withers basin sub-watersheds. Concentrations below the informal WQS of 2 mg DO/L are shaded dark green. The remaining samples were evenly divided into three groups. Sub-watersheds in gray reflect sites where no water was present during dry weather and hence no samples were collected.
Figure D.2.1-2. Turbidity (NTU) in Withers basin sub-watersheds. Concentrations below the SC DHEC WQS of 50 NTU are shaded green. Sub-watersheds in gray reflect sites where no water was present during dry weather and hence no samples were collected.
The average percentage that VSS comprised of TSS across all sites and events was 33%. Since bacteria tend to adhere to particles, the organic-rich nature of the sediment could be providing an ideal environment for the survival of FIB. As shown in Figure D.2.1-3, a substantial fraction of the variability in BOD5 can be attributed to VSS (p < 0.001) suggesting the particulate organic matter is in a form that is available for microbial metabolism.

**Figure D.2.1-3.** Relationship between BOD5 (mg DO/L) and VSS (mg/L) concentrations. All measurements for all sites are plotted. A significant linear correlation was observed.

**D.2.2. Human-specific chemical tracers**

Optical brighteners are additives in laundry detergents that make clothes look brighter. A significant level of these compounds in the environment is an indicator of wastewater discharges. The screening method of Cao et al. (2009) was used to identify significant levels of optical brighteners.\(^1\) Optical brighteners were detected at significant levels in 23 of the 54 samples measured by the Cao method. As shown in Figure D.2.2-1, Sites 4, 5, 6, 12, 13, and 14 had the strongest evidence for optical brighteners, with at least three of the samples at each of these sites meeting the screening criteria for significant detections.

\(^1\) This screening technique was not applied to the first dry weather sampling. The less specific method of Hartel et al. (2007) was used.
Figure D.2.2-1. Presence of optical brighteners. The red-shaded sub-watersheds denote a significant level of optical brighteners and green-shaded sub-watersheds had levels that were below concern. The Hartel et al. (2007) method was used on 5/20/2012. To increase sensitivity, all subsequent samplings utilized the Cao et al. (2009) method. Sub-watersheds in gray reflect sites where no water was present during dry weather and hence no samples were collected.
Caffeine was pilot tested as a chemical tracer of human-sourced fecal inputs during the last two wet weather sampling events. This chemical is a tracer of human wastewater as it is excreted in urine. Sauvé et al. (2012) have proposed a threshold concentration of >0.4 ng/mL as evidence for the presence of significant human fecal contamination. This threshold was derived from correlations between caffeine and fecal coliforms in natural waters. As shown in Figure D.2.2-2, caffeine was observed in some of the sub-watersheds at concentrations above the threshold proposed by Sauvé et al. (2012). This tracer is thus interpreted as supporting the presence of human inputs at sampling sites 3, 4, 8 and perhaps 7.
Figure D.2.2-2. Caffeine (ng/mL) in Withers basin sub-watersheds. Concentrations were grouped into quartiles. Sub-watersheds in gray reflect sites where no water was sampled. The only samples analyzed for caffeine were those collected during the final two wet weather events.
**D.2.3. Water Mass Tracers**

Specific conductivity, temperature, pH, and dissolved oxygen were measured to help determine where waters in the drainage system originate and their pathway through the watershed. See Appendix E for maps and graphs for these parameters.

These tracers are not necessarily specific for fecal inputs. Since some are elevated by the input of wastewaters, they are recommended for use by stormwater managers as tracers of illicit discharges, most notably specific conductivity (Pitt 2004). The specific conductivity at Site 3 approximately doubled following a sewer line break upstream.

Several sites had highly supersaturated levels of dissolved oxygen (>200%) during the dry weather sampling conducted on 5/20/12. On this date, these sites (5, 8 and 11) also had pH > 8.5 which represents a contravention of the Class FW WQS. The pH at site 8 was 9.5. Site 3 also had a pH > 8.5 on 5/20/13 and a somewhat lower but still supersaturated level of dissolved oxygen (125%). The supersaturated oxygen levels suggest a high degree algal productivity, which is an indicator of cultural eutrophication. A rapid rate of photosynthesis is consistent with elevated pH due to uptake of CO₂ which is a weak acid. None of the other chemical tracers were unusually high on these sites compared to other sampling dates. Measurements of other tracers for eutrophication were not made as part of this study, i.e. chlorophyll, nitrate, nitrite, orthophosphate, total nitrogen or total phosphorus, so the occurrence of an algal overgrowth cannot be confirmed. Nonetheless, an algal bloom is not inconsistent with fecal contamination as the associated organic matter contains nutrients that are released into the water and hence made available for uptake by the algae. This release process occurs as the fecal material decomposes so cultural eutrophication is a possible consequence of fecal contamination.

**D.2.4. Toxicity**

A broad screen for toxicity in the water samples was performed using a bioassay. The goal of this work was to provide background data for future efforts at addressing water quality concerns beyond FIB in Withers Swash. This bioassay employed water fleas (*Daphnia magna*) provided by Kingwood Diagnostics commercially as IQ-Tox™. (See [http://www.kingwooddiagnostics.com/](http://www.kingwooddiagnostics.com/) for more details on this bioassay.)

The IQ-Tox™ test is designed to detect biological toxins, such as botulinum toxin, ricin, and the nerve agents soman and VX. The organisms are also sensitive to pesticides, rodenticides, pharmaceuticals, and industrial chemicals. The organisms exhibit a detectable response to these toxins at concentrations substantially below human health thresholds and hence are used as a screening tool to protect against human health threats. Other chemicals that induce a detectable response are trace metals. These responses have been detected at concentrations of 0.65, 0.07, and 0.36 mg/L for copper, iron, and aluminum, respectively (James et al. 2003). CCU has measured a toxic threshold level of 0.1 mg/L for copper, 0.2 mg/L for lead, 0.02 to 0.04 mg/L for Zn, and 70 mg/L for ammonia. No toxicity was observed for chromium. The test organisms are also sensitive to salty waters (specific conductivity >11 mS/cm).

The results of IQ-Tox™ tests are shown in Figure D.2.4-1. They suggest widespread toxicity during both wet and dry sampling events. In contrast, only 11 of 882 bioassays (1%) conducted at 9 sites as part of a monitoring program in the nearby Waccamaw and Pee Dee Rivers have exhibited a toxic
Four of those 11 toxic responses were attributable to elevated levels of salt (specific conductivity > 11 mS/cm). This could explain the toxic responses observed at Site 12, the only tidally influenced site, where specific conductivity was always > 20 mS/cm.

The maximum ammonium concentrations observed in the Withers Basin were <0.5 mg N/L and are well below the toxic threshold of 70 mg N/L. The most recent available dissolved iron concentrations measurements were made on 8/8/06 and 8/24/06 at a downstream site (WAC-022AS1) in Withers Swash. These concentrations were 0.07±0.01 and 0.06±0.01 mg/L, respectively (B. Lewis, pers.comm.). This suggests that ambient iron concentrations might be the cause of at least some of the toxic response.

The only sampling event during which no toxicity was observed at any of the sites was the wet weather event conducted on 5/30/12. A possible explanation is that the heavy rains (~1.5”) during this event diluted the toxic agent(s). Although the conductivity at Site 12 exhibited its lowest value on 5/30/12, the level was still high (22 mS/cm). Nevertheless, no toxicity was observed at this site on that date.

Further investigations to determine the cause of the widespread toxic response are suggested. Potential toxic agents are suggested in Table C.3 (Appendix C) which lists storage issues for petroleum hydrocarbons and other industrial chemicals at many of the sampling sites. Run-off from roads is another potential source of toxics, including trace metals and polynuclear aromatic hydrocarbons (PAHs).

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2 The results of this monitoring program are available at: [http://bccmws.coastal.edu/river_gauge/](http://bccmws.coastal.edu/river_gauge/)
Figure D.2.4-1. Toxicity in Withers basin sub-watersheds as detected by a *Daphnia magna* bioassay. Green shading denotes non-toxic effects and red represents a finding of toxicity. Sub-watersheds in gray reflect sites where no water was present during dry weather and hence no samples were collected.
D.3 Weight of Evidence Index

To enable a weight-of-evidence approach in analyzing the data, indices were developed for each parameter as described in Section 3.4.3 for the FIB and genotypic assays results. These indices were tabulated in color-coded matrices to help visualize which sites had the largest fecal sources and under what conditions, “wet” vs. “dry” as well as which sites had detections for specific animal sources and which were strongest.

An index similar to the ones developed for the FIB and qPCR markers was developed to rank order the chemical source tracer results. The resulting rank orders were combined with the FIB and qPCR rank orders to provide a weight-of-evidence confirmation of the most likely host animal sources at sites with the highest degrees of fecal contamination. This is shown in Tables 3.5.1-1 and 3.5.1-2.

The following is a description of how the rank ordering of the chemical tracers and bioassay results was performed. First, all the water quality data were rank ordered into groupings as shown in Table D.3-1. In most cases, these groupings represent quartiles for the concentration range exhibited by each parameter across all samplings and sample sites. When a WQS was available, this was used to establish the threshold for the lowest rank order and the remaining samples subdivided into thirds. These values were used to create Table D.3-2 by averaging all the sample results (wet + dry) at each site. The resulting index values were color coded using the same protocol as with the FIB and genotypic assay matrices (Tables 3.4.1-2 and 3.4.3-2). High numbers and red colors suggest samples with chemical evidence consistent with fecal inputs.

In the case of specific conductivity and pH, high values in freshwater are consistent with a source of wastewater (Pitt 2004). Low values for dissolved oxygen saturation were assigned a high value as reflective of the influence of oxygen-demanding substances. Oxygen saturation values are the percent which the aqueous dissolved oxygen concentration comprises of the concentration that should be present if the water had achieved equilibrium with atmospheric oxygen gas levels at ambient temperatures. Values less than 60% are suggestive of significant levels of oxygen-demanding substances such as contributed by particulate organic matter, dissolved organic matter, and/or ammonia.
Table D.3-1. Concentration ranges used to assign rank orders to chemical tracer concentrations and toxicity assay results. The percentile range that each rank order encompasses is shown in parentheses. These rank order assignment were used to create Table D.3-2.

Table D.3-2. Matrix showing the average rank order values for each chemical tracer (wet + dry) as a function of location. The maximum average value for a single site is 4. Values are color coded from blue to green to yellow to orange to red in increasing order of average rank value.
D.4. Summary of Findings

Contraventions of several regulatory and informal water quality standards were observed during wet and dry sampling events. Most wet (37 of 42; or 88.1%) and dry (23 of 24; or 95.8%) weather samples had five-day biochemical oxygen demand (BOD5) exceeding an informal water quality standard of 2.0 mg/L. Almost one third of the samples (19 of 66; or 28.8%) had turbidities that contravened the SC DHEC water quality standard of 50 NTU for Class FW waters, particularly during wet weather events. High concentrations of total suspended solids (TSS) and volatile suspended solids (VSS), and turbidity were also observed. Ammonia concentrations were below levels toxic to aquatic organisms (EPA 2009).

High values of turbidity, ammonia, total suspended solids (TSS), volatile suspended solids (VSS), and 5-day biochemical oxygen demand (BOD5) are consistent with findings of high FIB concentrations because fecal matter imparts these chemicals to the water. Other sources of these chemicals can be present, so these tracers are best used in a weight-of-evidence context. This is provided in Tables 3.5.1-1 and 3.5.1-2. The tables document that all the chemical tracers were internally consistent with the FIB concentration results giving confidence to the site specific conclusions regarding the presence of human and canine sources of fecal contamination suggested by the genotypic assays.

The widespread toxic response exhibited by the IQ-Tox™ bioassay warrant further investigation to determine likely causes. This is support the development of CMB’s illicit discharge detection and elimination program required under the state’s NPDES Phase II stormwater permit.

D.5. References


