**Understanding Nutrient Impacts and Sources at the Watershed Scale to Enhance Environmental Stewardship**

Matthew Flood, Tiong Aw, Sherry Martin, and Joan B. Rose

December 30th, 2016

## EXECUTIVE SUMMARY

Our team focused on understanding the source of nutrients from crop land using new source tracking tools, watershed assessment, mapping and statistics. We were interested in nutrients, cow, pig and chicken markers, fertilizers as well as land use characteristics to identify key watersheds which might be impacted.

Our key findings were as follows:

1. All MST markers are stable enough to follow through three flow regimes at large watershed scales.
2. The chicken marker is the most variable and found at the highest concentrations, related to manure application.
3. High porcine MST was found in the southeast.
4. The MST markers increase in prevalence in the spring melt compared to base flow. The large watershed become positive during summer rains.
5. The MST concentrations generally get diluted during the summer rains, concentrations are highest in Spring Melt.
6. Key watersheds were identified where the MST markers were always present and others that had very high concentrations.
7. There were two watersheds where all markers were found in all three seasons, Sandy Creek in Monroe County and Tiltabawasee River which goes through Arenac, Bay, Clare, Gladwin, Gratiot, and Isabella counties. The Big Sable River, Elk Torch and Cheboygan watersheds were low for all three markers.
8. TP is related to rain.
9. Septic tanks are contributors of TP.
10. More Riparian zones as buffers without ag, septic tanks are related to less MST and nutrients.

## GOALS OF THE STUDY

**Objectives:** The overarching goal of this proposed research is to elucidate relationships between agricultural characteristics, nutrient loading, and sources at the watershed scale to resulting water quality across the entire lower peninsula of Michigan.

1. Analyze archived water samples for Bovine, Porcine, and Chicken source-specific DNA markers.

2. Identify associations between nutrients, microbial source tracking (MST) markers, land use, land characteristics, agricultural practices, and climate.

3. Create maps identifying locations to focus mitigation strategies.

**Background:** In the past it has not been possible to distinguish water quality impacts of fertilizer from those derived from human waste and animal waste/ manure. However, new microbial source tracking (MST) tools allow specific identification of fecal pollution impacted runoff. We are working on determining the sources of fecal pollution from 64 watersheds in the Lower Peninsula of Michigan under three flow regimes, in order to identify the impacts of agricultural management practices on water quality and provide guidance to focus efforts for water quality improvement across the state and surrounding agricultural regions.

## METHODS

A full description of the methods can be found in Appendix A. Briefly we studied 64 watersheds in the lower peninsula of Michigan. Water samples were collected during 3 seasons, fall, Spring melt and summer rain. These samples were tested for an array of chemical tests including for nutrients (total phosphorous, soluble reactive phosphorous, total nitrogen, nitrate and ammonia) animal markers, cow, pig and chicken to indicate manure and also land use and rain fall. The data were summarized using descriptive statistics (averages and ranges), markers, seasons and watershed compared. Maps were prepared.

1. The Cow M2 Bovine, Pig2 Bac Porcine and the CL Chicken marker's droplet digital PCR (ddPCR) cycles were optimized to provide more precision around the concentrations of the markers in the various watersheds contrasting the three flow conditions.

2. Quality assurance and quality control (QA/QC) guidelines were established for MST using ddPCR.
3. DNA was extracted from all statewide baseflow, spring melt and summer rain samples for analysis.
4. Analysis of all statewide baseflow, spring melt and summer rain DNA samples was completed for the M2P Bovine marker, Pig2 Bac Porcine marker and the CL Chicken marker.
5. Maps were completed for presence/absence and concentrations for the markers and the nutrients.
6. Statistical analysis using CART was completed.

## RESULTS

### Concentrations of the Bovine, Porcine, and Chicken source-specific DNA markers (objective 1).

Table 1 shows the percentage of samples positive, averages and ranges under the statewide baseflow, spring melt and summer rain for the three markers.

Under baseflow 45%, 59% and 64% of watersheds were positive for the Bovine, Porcine and Chicken markers, respectively with average concentrations of 46, 38 and 35 cell equivalents (CE)/100ml, respectively. In contrast during the statewide spring melt 59%, 72% and 81% of the watersheds were positive for the Bovine, Porcine and Chicken markers, respectively with average concentrations of 23, 27 and 170 CE/100ml, respectively. During the summer rain, 76%, 62% and 67% of the watersheds were positive for the Bovine, Porcine and Chicken markers, respectively with average concentrations of 31, 16 and 41 CE/100ml, respectively.

Different trends were seen with each marker reflecting use, applications and stability of the markers. The percentage of watersheds with the Bovine marker was lower during baseflow and increased with spring melt and summer rains. The percentage of watersheds with the Porcine marker was slightly higher than the Bovine during baseflow and also increased during spring melt but then decreased slight during summer rain. The Chicken marker was higher than all the markers but was slightly diluted during summer rains. (Figure 1). Figure 2 shows the shows the variability of the concentrations of the markers during the three seasons.
The chicken marker is the most variable but has the highest median value.
The bovine marker concentration is fairly low until the summer rains.
All marker concentrations on average increase during the summer rains.

**Table 1. Bovine, Porcine and Chicken Markers Occurrence and Concentrations in the Watersheds in the Lower Peninsula of Michigan**

| | Bovine | | | Porcine | | | Chicken | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Baseflow** | **Spring Melt** | **Summer Rain** | **Baseflow** | **Spring Melt** | **Summer Rain** | **Baseflow** | **Spring Melt** | **Summer Rain** |
| **Percentage of samples positive (# +/ total)** | 45.3% (29/64) | 59.4% (38/64) | 76.2% (48/63) | 59.4% (38/64) | 72% (46/64) | 62% (39/63) | 64.1% (41/64) | 81.3% (52/64) | 66.7% (42/63) |
| **Geometric Mean CE/100mL** | 9.00 | 5.09 | 9.27 | 6.58 | 11.36 | 5.81 | 6.56 | 28.17 | 10.91 |
| **Average CE/100mL** | 45.61 | 22.54 | 31.40 | 37.64 | 26.76 | 15.85 | 35.21 | 170.96 | 40.89 |
| **Range CE/100mL** | 2.09 to 697.2 | 1.60 to 582.4 | 1.60 to 612.8 | 1.93 to 840 | 1.93 – 253.6 | 1.93 to 370.4 | 1.93 to 1084 | 1.93 to 1688 | 1.93 to 246.4 |

CE is Cell equivalents; Detection limit was 1.28

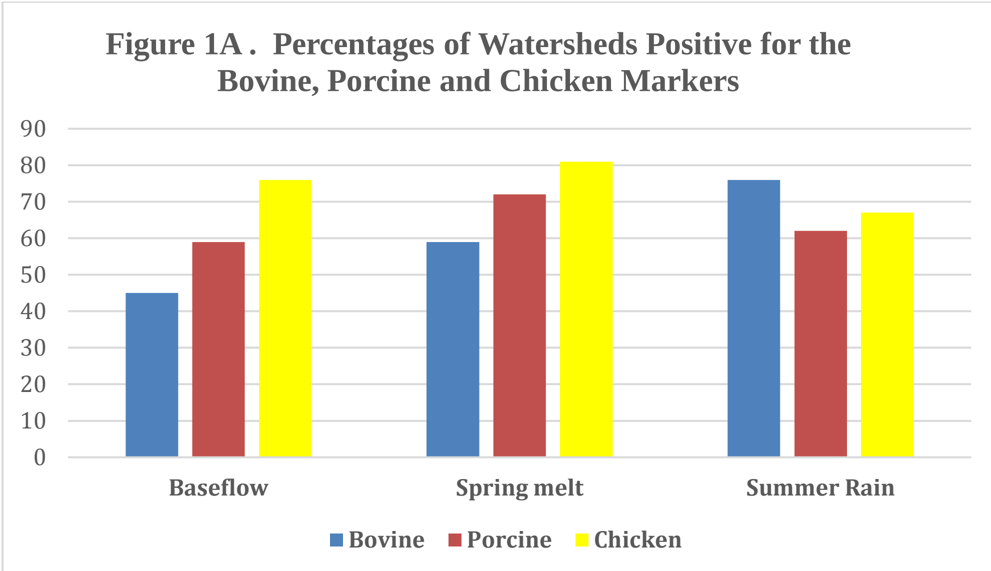
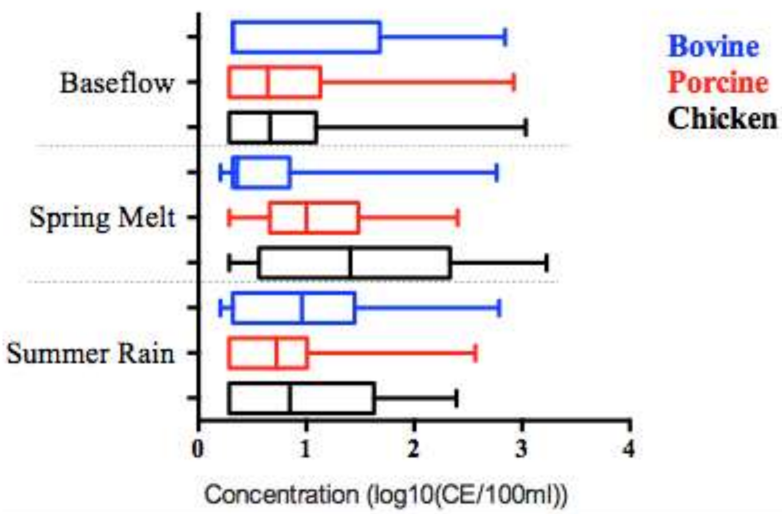


**Figure 1A . Percentages of Watersheds Positive for the Bovine, Porcine and Chicken Markers**

**Figure 1B. Concentrations of the Markers in the three Seasons (shown as box plots with medians, and 50 percentiles and 95% confidence limits).**




Overall, while the averages were not statistically different, the percent positives increased as more overland flow increased for the Bovine marker. The Chicken marker was there at the highest percentage but decreased slightly with the summer rains as did the Porcine marker. All markers increased from baseflow to Spring melt.

The markers and nutrients did not correlate to each other. Table 2 shows the r values. This suggests that the increase in nutrients was not related to the increase in markers either do to the sources being different (eg fertilizer versus manure) or that the transport was different which is to be expected as bacteria move as particles in water and nutrients are dissolved chemicals.

Table 2 Correlation coefficients between MST markers and nutrients.

| LOG10 | TN | TP | NOX | NH3 | SRP | Bovine | Porcine | Chicken |
|---|---|---|---|---|---|---|---|---|
| TN | 1 | 0.58 | 0.67 | 0.28 | 0.33 | -0.12 | 0.16 | 0.17 |
| TP | 0.58 | 1 | 0.50 | 0.30 | 0.50 | -0.15 | 0.23 | 0.25 |
| NOX | 0.67 | 0.50 | 1 | 0.24 | 0.42 | -0.15 | 0.24 | 0.16 |
| NH3 | 0.28 | 0.30 | 0.24 | 1 | 0.17 | -0.14 | 0.06 | 0.14 |
| SRP | 0.33 | 0.50 | 0.42 | 0.17 | 1 | -0.11 | 0.16 | 0.07 |
| Bovine | -0.12 | -0.15 | -0.15 | -0.14 | -0.11 | 1 | 0.03 | 0.15 |
| Porcine | 0.16 | 0.23 | 0.24 | 0.06 | 0.16 | 0.03 | 1 | 0.22 |
| Chicken | 0.17 | 0.25 | 0.16 | 0.14 | 0.07 | 0.15 | 0.22 | 1 |

Based on the detection of the Bovine, Porcine and Chicken MST markers the watersheds were categorized as highly impacted (all three sampling times, baseflow, spring, summer were positive for the watershed), moderately impacted (two of the three were positive) or less impacted (where one or none of the samples were positive). The results are shown in Table 3, where 27%, 34% and 39% of the watersheds were placed in the highly impacted category for Bovine, Porcine and Chicken MST. This was somewhat independent of size of the watershed however the larger watersheds seem to have somewhat lower marker due likely to dilution.

There were two watersheds where all markers were found in all three seasons, Sandy Creek in Monroe County and Tiltabawasee River which goes through Arenac, Bay, Clare, Gladwin, Gratiot, and Isabella counties. The Big Sable River, Elk Torch and Cheboygan watersheds were low for all three markers.

**Table 3: Watershed Impacted by the Bovine, Porcine and Chicken MST Markers (as shown by size of the watershed along with representative Counties) High impact is considered when the marker was present for all three seasonal samples.**

| Watershed | Size of the Water-Shed Km$^2$ | Bovine Impact | Porcine Impact | Chicken Impact | # of MST high | Counties |
|---|---|---|---|---|---|---|
| Buck Creek | 2.9 | Mode-rate | HIGH | Low | 1 | Kent |
| Little Pigeon Creek | 14 | HIGH | HIGH | Low | 2 | Ottawa |
| Belangers Creek | 25 | HIGH | Low | HIGH | 2 | Leelanau |
| Monroe Creek | 27 | HIGH | Moderate | Low | 1 | Antrim, Charlevoix |
| Little Trout River | 28 | HIGH | Moderate | HIGH | 2 | Presque Isle |
| Mitchell Creek | 38 | Mode-rate | HIGH | HIGH | 2 | Grand Traverse |
| Silver Creek | 41 | HIGH | Moderate | Mode-rate | 1 | Muskegon |
| Pine Creek | 48 | Mode-rate | HIGH | Mode-rate | 1 | Ottawa |
| Harrington Drain | 53 | HIGH | Low | HIGH | 2 | Macomb, Wayne |
| Swan Creek | 54 | Mode-rate | HIGH | HIGH | 2 | Mason |
| Marsh Creek | 78 | Mode-rate | Moderate | HIGH | 1 | Wayne |
| Flower Creek | 79 | Mode-rate | HIGH | Low | 1 | Muskegon, Oceana |
| Sandy Creek | 82 | HIGH | HIGH | HIGH | ALL | Monroe |
| Trout River | 82 | HIGH | Moderate | HIGH | 2 | Presque Isle |
| Pigeon River | 102 | Mode-rate | HIGH | HIGH | 2 | Ottawa |
| Crystal River | 110 | Mode-rate | Low | Mode-rate | 0 | Leelanau |
| Carp River | 119 | HIGH | Moderate | HIGH | 2 | Cheboygan, Emmet |
| Bass River | 127 | Mode-rate | HIGH | Mode-rate | 1 | Ottawa |
| Black Creek | 136 | Mode-rate | HIGH | Low | 1 | Muskegon, Newaygo |
| Sand Creek | 142 | Mode-rate | HIGH | Low | 1 | Kent, Ottawa |
| Rush Creek | 152 | HIGH | Moderate | HIGH | 2 | Kent, Ottawa |
| Stony Lake Outlet | 160 | Mode-rate | HIGH | HIGH | 2 | Oceana |
| Long Lake Creek | 162 | Mode-rate | Low | HIGH | 1 | Alpena, Presque Isle |
| Jordan River | 174 | Mode-rate | Low | Mode-rate | 0 | Antrim, Charlevoix |

| | | | | | | |
|---|---|---|---|---|---|---|
| Boyne River | 199 | HIGH | **Moderate** | **Mode-rate** | 1 | Antrim, Charlevoix, Otsego |
| Lincoln River | 215 | HIGH | HIGH | Low | 2 | Mason |
| Macatawa River | 292 | Low | HIGH | Low | 1 | Allegan, Ottawa |
| Bear River | 293 | **Mode-rate** | Low | HIGH | 1 | Charlevoix, Emmet |
| South Branch Black River | 313 | **Mode-rate** | **Moderate** | HIGH | 1 | Allegan, Van Buren |
| Bear Creek | 350 | **Mode-rate** | Low | **Moderate** | 0 | Benzie, Manistee, Wexford |
| Ocqueoc River | 369 | **Mode-rate** | **Moderate** | HIGH | 1 | Montmorency, Presque Isle |
| North Branch Black River | 398 | Low | HIGH | **Mode-rate** | 1 | Allegan, Van Buren |
| Tawas River | 403 | HIGH | **Moderate** | HIGH | 2 | Iosco |
| Pine River | 440 | Low | HIGH | **Mode-rate** | 1 | Macomb, St. Clair |
| Platte | 471 | **Mode-rate** | Low | Low | 0 | Benzie, Grand Traverse, Leelanau |
| Big Sable River | 476 | Low | Low | Low | all low | Lake, Manistee, Mason |
| Belle River | 512 | **Mode-rate** | HIGH | **Mode-rate** | 1 | Lapeer, Macomb, Oakland, St. Clair |
| Little Manistee | 526 | **Mode-rate** | **Moderate** | HIGH | 1 | Lake, Manistee, Mason, Wexford |
| Betsie | 618 | **Mode-rate** | **Moderate** | **Mode-rate** | 0 | Benzie, Grand Traverse, Manistee |
| Boardman | 716 | Low | Low | **Mode-rate** | 0 | Grand Traverse, Kalkaska |
| Rifle | 858 | Low | **Moderate** | HIGH | 1 | Arenac, Gladwin, Ogemaw, Roscommon |
| Au Gres | 987 | **Mode-rate** | Low | **Mode-rate** | 0 | Arenac, Iosco, Ogemaw |
| Paw Paw River | 1027 | **Mode-rate** | HIGH | HIGH | 2 | Berrien, Kalamazoo, Van Buren |
| River Rouge | 1033 | **Mode-rate** | HIGH | HIGH | 2 | Oakland, Washtenaw, Wayne |
| White River | 1049 | Low | **Moderate** | HIGH | 1 | Muskegon, Newaygo, Oceana |
| Black River | 1250 | HIGH | **Moderate** | **Mode-rate** | 1 | Lapeer, St. Clair, Sanilac |
| Elk-Torch | 1308 | Low | Low | Low | all low | Antrim, Charlevoix, Grand Traverse, Kalkaska, Otsego |
| Black | 1509 | **Mode-rate** | Low | Low | 0 | Cheboygan, Montmorency, Otsego, Presque Isle |
| Shiawassee River | 1517 | **Mode-rate** | **Moderate** | **Mode-rate** | 0 | Genesee, Livingston, Oakland, Saginaw, Shiawassee |
| Pere Marquette | 1790 | Low | Low | **Mode-rate** | 0 | Lake, Mason, Newaygo, Oceana |
| Clinton River | 1880 | **Mode-rate** | **Moderate** | HIGH | 1 | Lapeer, Macomb, Oakland, St. Clair, Wayne |
| Cass River | 2174 | HIGH | Low | **Mode-rate** | 1 | Genesee, Huron, Lapeer, Saginaw, Sanilac, Tuscola |

| | | | | | | |
|---|---|---|---|---|---|---|
| Thunder Bay | 2241 | **Mode-rate** | **Moderate** | Low | 0 | Alcona, Alpena, Montmorency, Oscoda, Presque Isle |
| Huron | 2298 | **Mode-rate** | **Moderate** | **Mode-rate** | 0 | Ingham, Jackson, Livingston, Monroe, Oakland, |
| Cheboygan | 2317 | Low | Low | Low | all low | Charlevoix, Cheboygan, Emmet, Otsego |
| Raisin | 2683 | **Mode-rate** | Low | HIGH | 1 | Hillsdale, Jackson, Lenawee, Monroe, Washtenaw |
| Flint River | 3206 | **Modera te** | Low | **Mode-rate** | 0 | Genesee, Lapeer, Oakland, Saginaw, Sanilac, Tuscola |
| Manistee | 3559 | Low | Low | **Mode-rate** | 0 | Antrim, Benzie, Crawford, Grand Traverse, Kalkaska, |
| Kalamazoo | 5002 | **Modera te** | HIGH | **Mode-rate** | 1 | Allegan, Barry, Calhoun, Eaton, Hillsdale, Jackson, |
| Au Sable | 5287 | HIGH | Low | Low | 1 | Alcona, Crawford, Iosco, Kalkaska, Montmorency, |
| Tiltabawasee River | 6211 | HIGH | HIGH | HIGH | ALL | Arenac, Bay, Clare, Gladwin, Gratiot, Isabella, |
| Muskegon | 6418 | Low | **Moderate** | **Mode-rate** | 0 | Clare, Crawford, Kalkaska, Lake, Mecosta, Missaukee, |
| St. Joseph | 11061 | Low | HIGH | **Mode-rate** | 1 | Berrien, Branch, Calhoun, Cass, Hillsdale, Kalamazoo, |
| Grand | 12854 | Low | **Moderate** | **Mode-rate** | 0 | Allegan, Barry, Calhoun, Clinton, Eaton, Gratiot, |

**The associations between nutrients, MST markers, land use, land characteristics, agricultural practices, and climate (objective 2).**

Table 4 shows the data on the nutrients for the 64 watersheds. Phosphorous goals for the Great Lakes is 7 ug/Liter and NPDES permits from wastewater treatment is 1000 ug/L for nutrients. On average one can see that total phosphorous is over the Great Lakes goal but is below the discharge limits for sewage effluents. The nutrient concentrations for spring melt are highest, followed by summer rains and are lowest during baseflows.

**Table 4. Nutrients Ranges and (Averages) in ug/liter in the Statewide study over three seasons.**

| | TP ug/L | SRP ug/L | TN ug/L | NH3 ug/L | NOx ug/L |
|---|---|---|---|---|---|
| Baseflow | 8-395 (38) | 0.9-266 (23) | 82-5583 (1082) | 0-280 (24) | 0-5639 (858) |
| Spring Melt | 9-513 (97) | 3-336 (27) | 253-7886 (1903) | 0.7-387 (87) | 2-6870 (1518) |
| Summer rain | 11-112 (48) | 1-36 (11) | 126-7776 (1276) | 2.0-730 (43.5) | 4-5682 (872) |

Statistical analyses were undertaken via classification and regression tree (CART) techniques. CART is a statistical method that splits the dependent variable (nutrients and MST markers) into homogeneous categories based on the influence of independent variables (landscape, agricultural, and climatic characteristics).

All CART analyses included the following variables:

1. Land Use/Cover: 5yr average CDL[1] , NLCD[2] 2011, Imperviousness
2. Landscape application of N & P from manure and chemical ag fertilizer (model kg/hectare)
3. Septic (model): count, density, population served
4. Soil hydraulic conductivity
5. Dams: count, density, avg and stdev of discharge and residence time
6. Waste Water Treatment Plants: discharge, population served
7. Population density
8. Prior precip: 6, 12, 18, 24 hours, and 2, 3, 4, 6, 8, days
9. Specific to sample collection day and time
10. Measured water temp and dissolved oxygen
11. Most summarized in watershed and 60m buffer zone
12. Land cover classes with less than 1% across the sheds (many CDL classes and Barren class from NLCD etc) were not included

[1]The CDL crop land data layer crop-specific land cover data layer created annually for the continental United States using moderate resolution satellite imagery and extensive agricultural ground truth. All historical CDL products are available for use and free for download through CropScape.. [2] National Land Cover Data provides spatial reference and descriptive data for characteristics of the land surface such as thematic class (for example, urban, agriculture, and forest), percent impervious surface, and percent tree canopy cover.

The CART graphics are included in appendix B. The main findings are shown in the following tables 5, 6 and 7 (baseflow, spring melt and summer rain)

The relationships between nutrients (nitrogen and phosphorus) and the Bovine, Porcine and Chicken markers are not clear. This is likely due to transport and fate issues.

Further analysis using classification and regression trees (CART) shows that other factors have a stronger relationships to the marker concentrations than nutrients. For example, CART analysis for Baseflow showed that the bovine marker is related to several landscape characteristics, specifically woody wetlands, pasture and all wetland types. CART results for spring melt also shows that landscape features are more strongly related to bovine marker concentrations than nutrients, with fallow cropland and planted fields (with RYE for that particular land use data set) as the particular land cover types. Summer rain CART results were quite different, with septic systems, preceding rainfall, soil hydraulic conductivity and chlorophyll a with the strongest relationships to the bovine marker.

These data suggest that the markers are related to the land use, is found when it rains and coming in from runoff. The relationship to septic tanks may be indicative of rural watersheds. In previous work we found that *E.coli* cultivatable concentrations did have a relationship to phosphorous suggesting some relationship to fecal sources and both can accumulate in soils or sediments and later be released.

It is not surprising that many sources are associated with the markers and nutrients. The relationships between the markers, nutrients and land characteristics show the following:

Interestingly septic tanks appeared to be a source of Phosphorous, generally agricultural lands, corn and soy, found during spring melt and summer rain. .
Not surprisingly precipitation was associated with the MST markers and nutrients. However no clear number of rainy days was related to these constituents. Less riparian buffers was also associated with more nutrients.
During base flow modeled application of fertilizer was related to TN and N-manure was related to the chicken marker.

**Table 5 Baseflow CART Results of the Top Variables Explaining the Dependent Variables**

| More Bovine Marker | More Porcine Marker | More Chicken Marker | More TN | More NOX | More TP |
|---|---|---|---|---|---|
| More Wetlands | Less mixed Forest | Less septic tanks<br>Less precipitation | More low intensity urban | More agriculture in the buffer | More total septic tanks in the 60 m buffer (riparian zones) |
| Less hay; non-alfalfa | More Herbaceous wetlands | Less Herbaceous wetlands | Less 6 day precip | Less grass land | Less shrubs |
| More grassland/pasture | Lower temperature | More N-manure applications | More N-fertilizer applications | Less medium intensity urban | |
| | More precipitation in 3 days | | More hay and non-alfalfa | | |
| | Less Corn | | | | |

TN total nitrogen; NOX nitrate and nitrite; TP total phosphorous

**Table 6 Spring Melt CART Results of the Top Variables Explaining the Dependent Variables**

| More Bovine Marker | More Porcine Marker | More Chicken marker | More TN | More NOX | More TP |
|---|---|---|---|---|---|
| Greater dissolved oxygen<br>Less septic tanks | More Precipitation at 4 days & 3 and 2 days | Less surface water | More Ag in the watershed | More Ag in the watershed | More soy beans |
| More dams | Less urban in the riparian zone | Less pasture (10% of the watershed) | Less grass lands | Less mixed forest | Less mixed forest |
| Less riparian forest<br>Less riparian septics | More 6 day precip | More low intensity urban | More agriculture in the buffer (riparian zone) | More corn | More corn |
| | More herbaceous wetlands | in watersheds with only 2% Pasture | | | |
| | | More water arteries in the riparian zone | | | |

TN total nitrogen; NOX nitrate and nitrite;TP total phosphorous;

**Table 7. Summer Rain CART Results of the Top Variables Explaining the Dependent Variables**

| More Bovine Marker | More Porcine Marker | More Chicken Marker | More TN | More NOX | More TP |
|---|---|---|---|---|---|
| Less septic tanks<br>More grass lands; herbaceous fields | Lower temperature | Less Mixed forest | More riparian agriculture | More riparian agriculture | Less open land grasses |
| More woody wetlands | More precipitation at 8 days | More herbaceous wetlands | More pasture and hay | Less riparian wetlands | More 8 day precip |
| More total precipitation in the prior 3 days | Less precip at 48 mm | Less Cultivatible crops<br>More forest | | More N-manure application | More agriculture in the watersheds |
| Greater hydrologic conductivity (soil) | More riparian septics | | | | More septic tanks |

TN total nitrogen; NOX Nitrate and nitrite; TP total phosphorous

**Watershed maps for Animal Markers and Nutrients To Assist in the Prioritization of Mitigation Strategies (objective 3)**

Maps were prepared for each marker for each season and for the nutrients. Both presence absence and concentrations demonstrate key watersheds with high concentrations.

The prevalence goes up as it rains as one can see from the spread of red positive watersheds during spring melt and summer rain. However the concentrations in key watersheds are high during base flow and decrease during spring melts. The summer rain samples were taken after fertilization and planting thus one can see how the concentrations increase across many watersheds.

**Figure 2. The Prevalence of the Bovine MST marker mapped by watershed under three flow regimes**

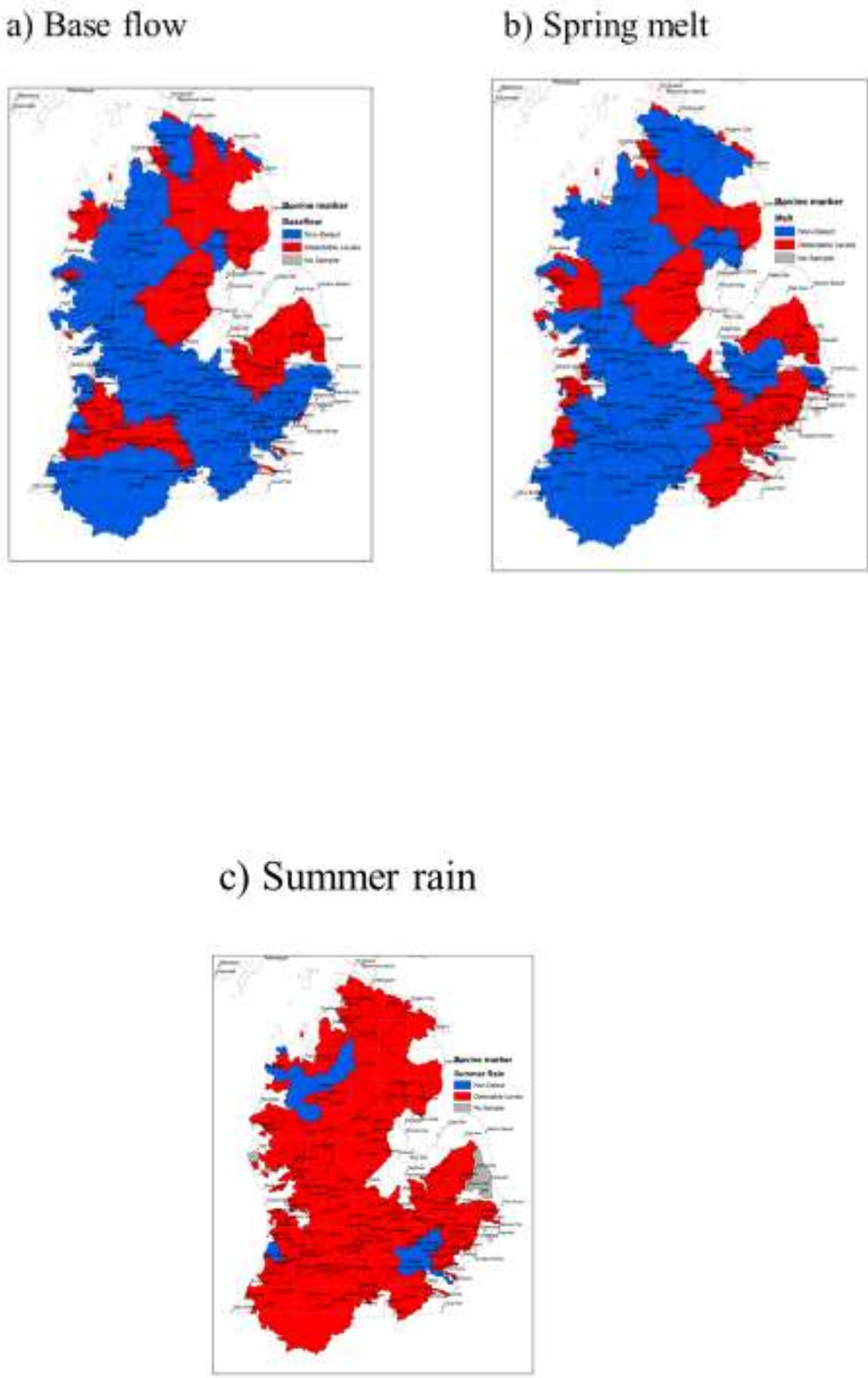
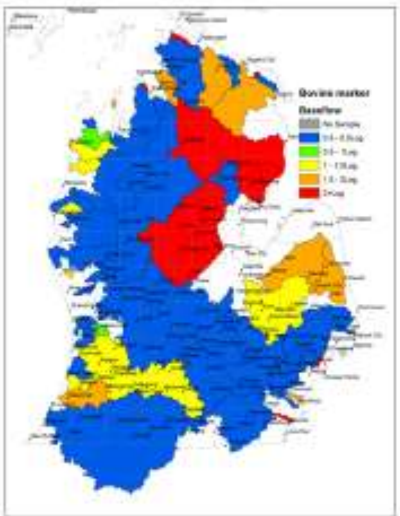
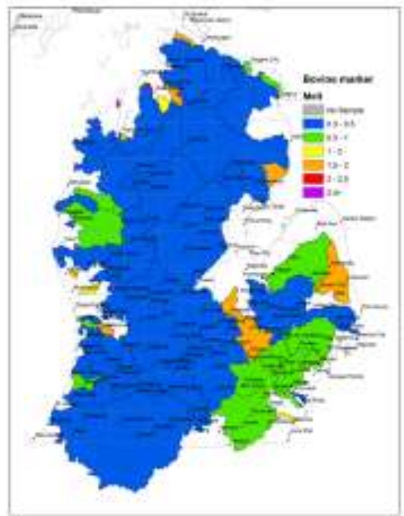


**Figure 3. The Concentration of the Bovine MST marker mapped by watershed under three flow regimes**

a) Base flow




b) Spring melt




c) Summer rain

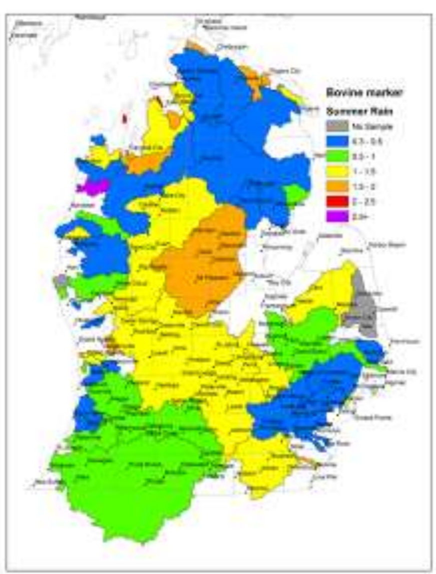


**Figure 4. The Prevalence of the Porcine MST marker mapped by watershed under three flow regimes**

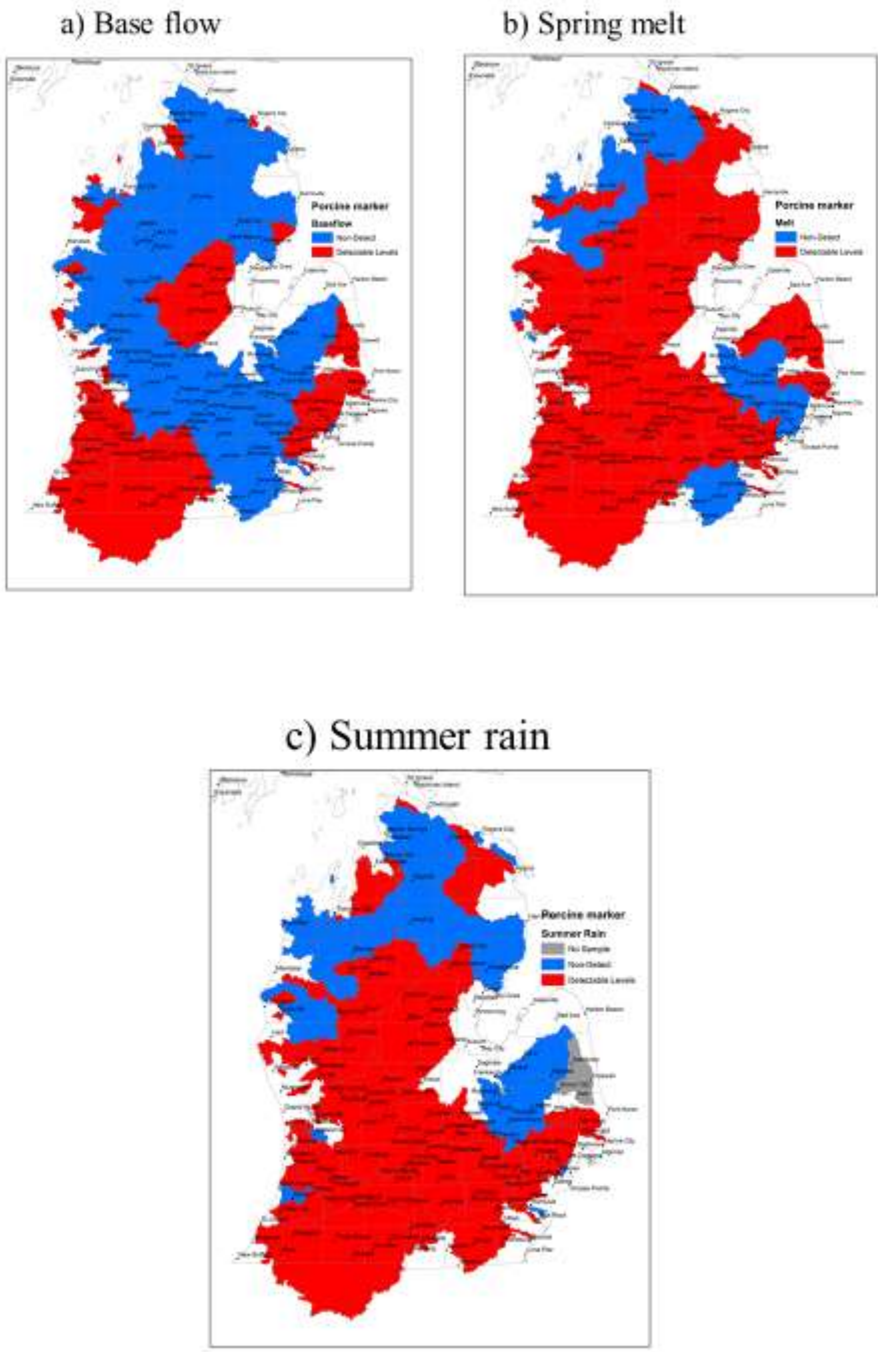


**Figure 5 The Concentrations of the Porcine MST marker mapped by watershed under three flow regime**

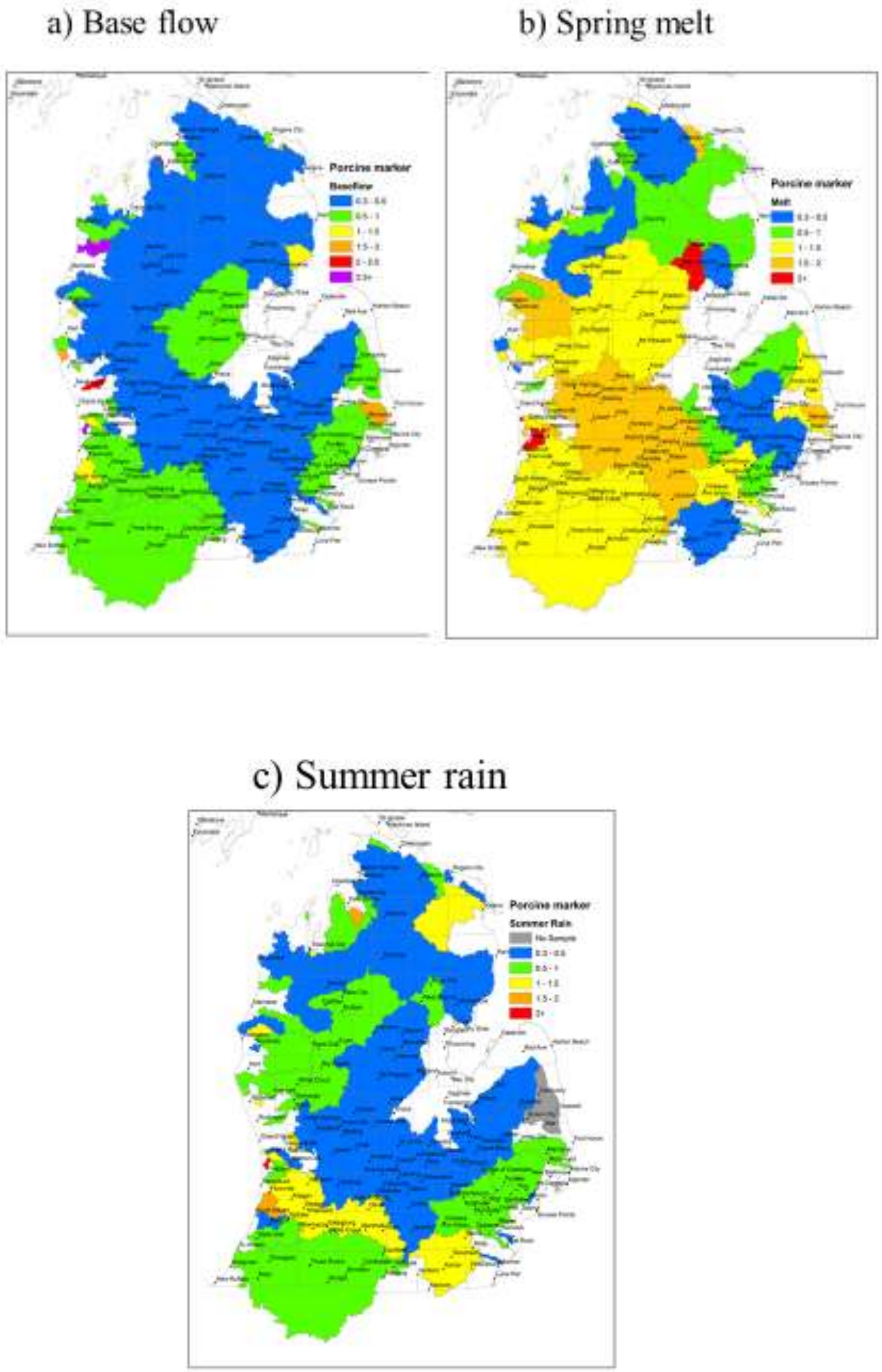
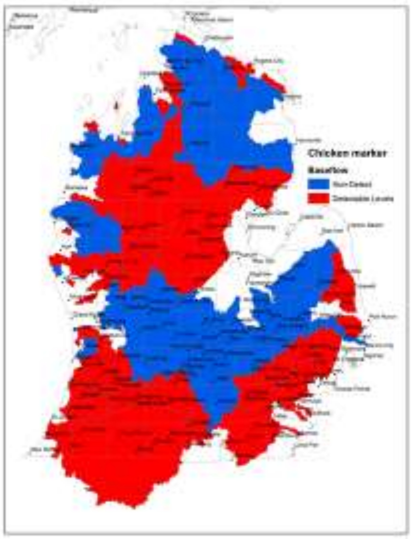
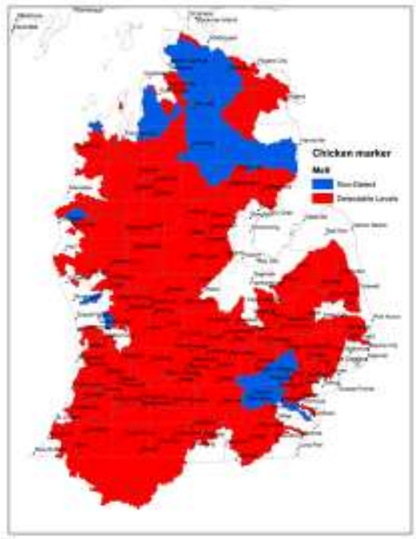


**Figure 6. The Prevalence of the Chicken MST marker mapped by watershed under three flow regimes**

a) Base flow




b) Spring melt




c) Summer rain

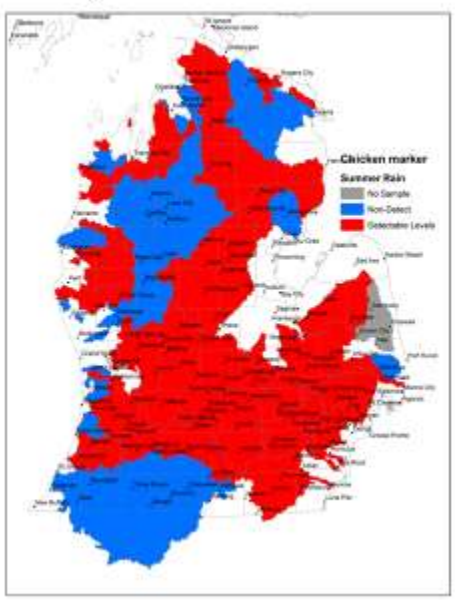
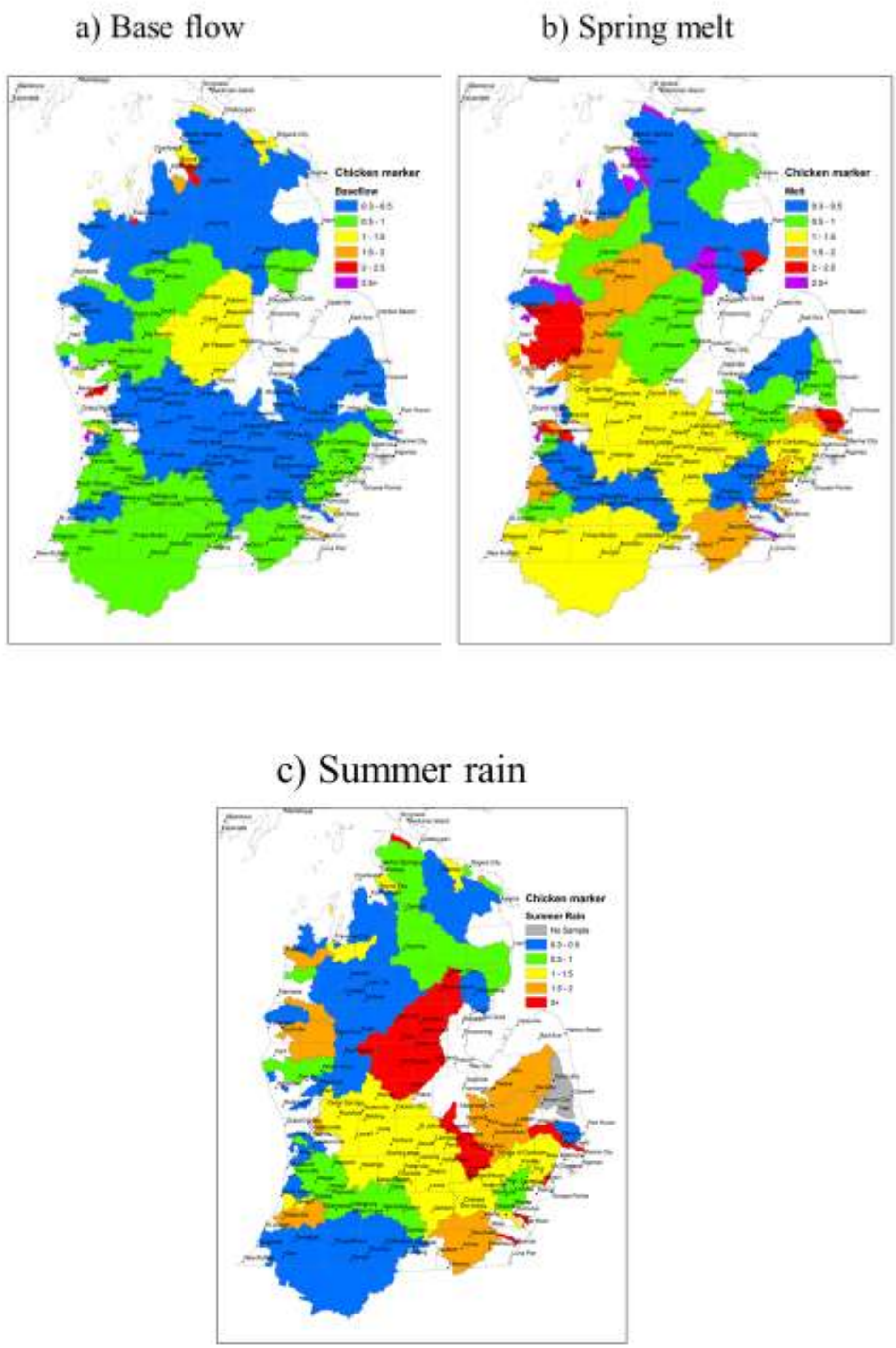
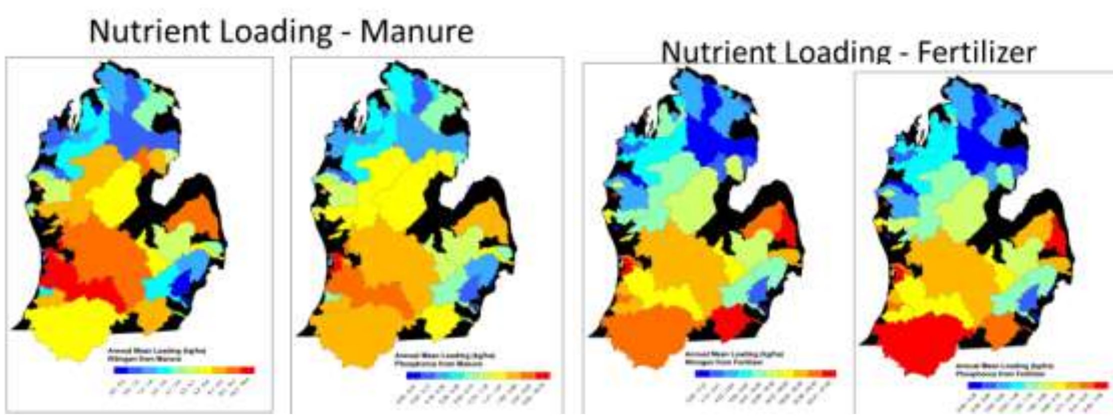


**Figure 7. The concentrations of the Chicken MST markers mapped by watershed under three flow regimes**



Figures 8 -10  Nutrient annual loading (Total nitrogen on the left and total phosphorous on the right in Kg/ hectare) demonstrate that the western part and very southern part of the state of MI are more impacted by manure where as the eastern part of the state is impacted by fertilizer.   The manure area overlaps with the pig farms in the area.



## CONCLUSIONS

1. All MST markers are stable enough to follow through three flow regimes at large watershed scales.
2. The MST markers increase in prevalence in the spring melt compared to base flow. The large watershed become positive during summer rains.
3. The MST concentrations generally get diluted during the summer rains, concentrations are highest in Spring Melt.
4. Key watersheds were identified where the MST markers were always present and others that had very high concentrations.
5. TP is also related to rain.
6. Septic tanks are contributors of TP.
7. More Riparian zones as buffers without ag, septic tanks are related to less MST and nutrients.

**APPENDIX A Methods and Procedures (Matthew Flood)**

**Study Area and Site Selection:**

The study area spans the watersheds that drain the vast majority of Michigan's Lower Peninsula to the Great Lakes. The watersheds were chosen as a part of a human sewage marker assessment project using the following criteria: 1) 30 large watersheds represent 65% of land area of the Lower Peninsula; and 2) the remaining 34 smaller watersheds were randomly selected around the state. Smaller watersheds were further filtered in the field to ensure sites that could be easily sampled and provide representative samples of the watershed rather than local or backwater conditions. In total, the 64 chosen river systems represent 84% of Michigan's Lower Peninsula drainage area based on our analysis of flow data and models. All sampling locations were located at bridge crossings that: are reasonably accessible, have adequate flow to be accurately measured using an Acoustic Doppler Current Profiler or a Marsh McBirney Flow meter, and do not have significant backwater effect from the Great Lakes at the lowest drainage points of the 64 watersheds examined. These samples were collected during three flow regimes (Baseflow, spring snow melt, and summer rain) by Verhougstraete *et al.* (2015). These samples were then concentrated from 1L to 2ml and stored at -80°C until DNA extraction was performed.

**Microbial Source Tracking analysis:**

The markers used are shown in Table A1.

| Table A1 - Summary of oligonucleotide primer and probes for ddPCR assays | | | |
|---|---|---|---|
| **MST Target** | **Assay** | **Primer and Probe Sequences (5' to 3')** | **Reference** |
| Bovine | Cow M2 | M2F: CGGCCAAATACTCCTGATCGT | Shanks *et al.*, 2008 |
| | | M2R: GCTTGTTGCGTTCCTTGAGATAAT | |
| | | M2P: FAM-AGGCACCTATGTCCTTTACCTCATCAACTACAGACA-BHQ | |
| Porcine | Pig2Bac | Pig2Bac41F: GCATGAATTTAGCTTGCTAAATTTGAT | Mieszkin et al., 2009 |
| | | Pig2Bac163Rm: ACCTCATACGGTATTAATCCGC | |
| | | Pig2Bac113MGB: FAM-TCCACGGGATAGCC-BHQ | |
| Chicken | CL | CLF: CCCGGGAAACTGGGTCTAAT | Ryu et al., 2014 |
| | | CLR: CCATCCCCAATCGAAAAACTT | |
| | | CLP: FAM- CCGGATACGACCATCTGCCGCA -BHQ | |

For this study 1 ml of the 2 ml of stored sample was transferred to a 1.5ml microcentrifuge tube and cell pelleted. Then the supernatant was removed and 20 µl of proteinase k, and 200 µl of lysing buffer were added to the remaining cell pellet. DNA extraction was performed using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The following is a brief description of the manufacturer's protocol. The microcentrifuge tube was then incubated at 56°C for 30 min followed by 95°C for 15 min in a waterbath. After incubation, the microcentrifuge tubes were centrifuged briefly and then 200 µl of 100% ethanol were added to the sample. This was then mixed by pulse-vortexing for 15 s and then briefly centrifuged to remove any remaining droplets of sample from the inside of the tube's lid. This mixture was then pipetted into a QIAmp spin column, and centrifuged at 6000 x g for 1 min. The column was then placed into a clean collection tube, while the filtrate was discarded. Next, 500µl of a wash buffer was added to the column and centrifuged at 6000 x g for 1 min. This filtrate was again discarded and the column was placed into a clean collection tube where 500 µl of a second wash buffer was added to the column and centrifuged at 20,000 x g for 3 min. The column was then place in another clean collection tube and centrifuged at 20,000 x g for 1 min. Finally the column was placed into a sterile microcentrifuge tube, and was eluted with 100 µl of AE buffer by centrifugation at 6000 x g for 1 min after a 3 min incubation at room temperature. After extraction, DNA samples were stored at -20°C until analysis.

**Digital PCR**

Samples were run in duplicate on the droplet digital PCR with at least two no template controls (NTC) samples present in each run.

Each reaction mixture contained 11 µl of 2x PCR Supermix, 10 mM of each primer,10 µM probe, 1 µl of PCR-grade $H_2O$, 5.5 µl of sample DNA. Primer and probe sets for bovine, porcine, and chicken MST were chosen from multiple peer-reviewed studies (Shanks *et al.*, 2008, Mieszkin *et al.*, 2009, Ryu *et al.*, 2014). The reaction mixture was then combined using microfluidics with droplet generation oil (20 µl of reaction mixture with 70 µl of oil) in a DG8 cartridge (Bio-Rad) using the Droplet Generator (Bio-Rad, California, USA). Following droplet generation the newly formed droplets were transferred to a 96-well PCR plate, heat-sealed using a foil plate seal (Bio-Rad), and then placed in a Bio-Rad iCycler thermocycler for PCR amplification with the following conditions. Initial denaturation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 58.9°C for 1 min, and a final cycle of 98°C for 10 min. After PCR amplification, the plate was transferred to the Bio-Rad Droplet Reader for analysis (fluorescence reading for each droplet in each well) using the RED (rare event detection) setting.

All assay used the following QA/QC requirements. Each sample had to have more than 10,000 accepted droplets. If a sample had less than three positive droplets after the threshold for positive and negative droplets was set then this sample was considered to be a non-detect (ND), and is to be reported as having zero positive droplets. The Threshold for positive and negative droplets was set at one standard deviation above the negative droplets (As determined by calculating the standard deviation from each run's NTC raw amplitude data).

**Water Chemistry**

Results from a total of 192 samples taken during baseflow, snow melt, and spring rain were analyzed following standard methods for ammonia, calcium, chloride, dissolved organic carbon, magnesium, nitrate/nitrite, chlorophyll a, potassium, sodium, soluble reactive phosphorus, sulfate, total dissolved phosphorus, total dissolved nitrogen, total phosphorus, total nitrogen. Water temperature (°C), specific conductance (ms cm-1), and dissolved oxygen (mg l-1) were measured in situ at each site using a YSI multi-probe.

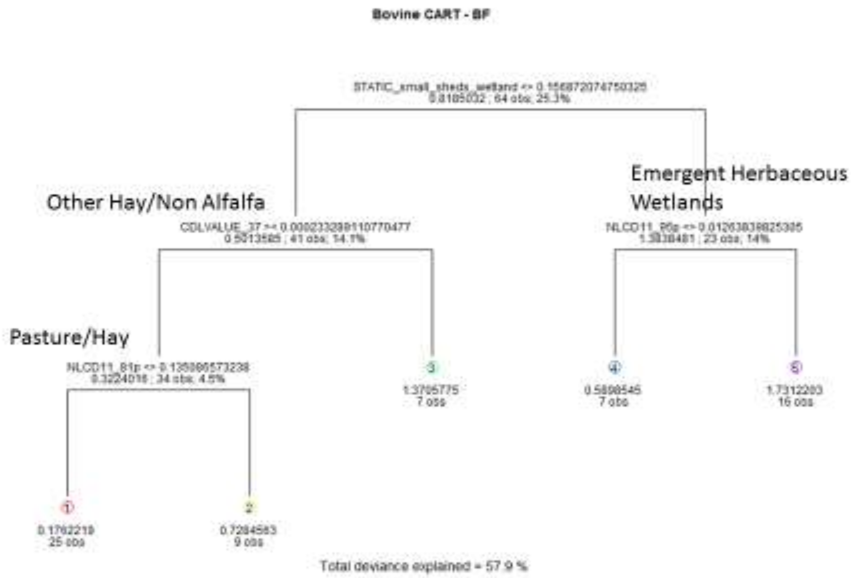
**Linking the Land to Nutrients and Sources:**

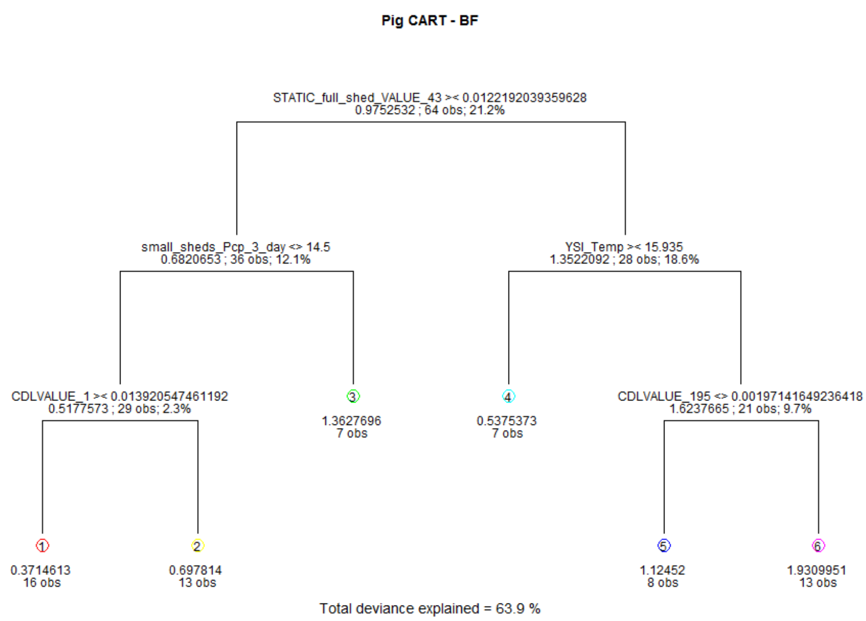
***Statistical Approach.***

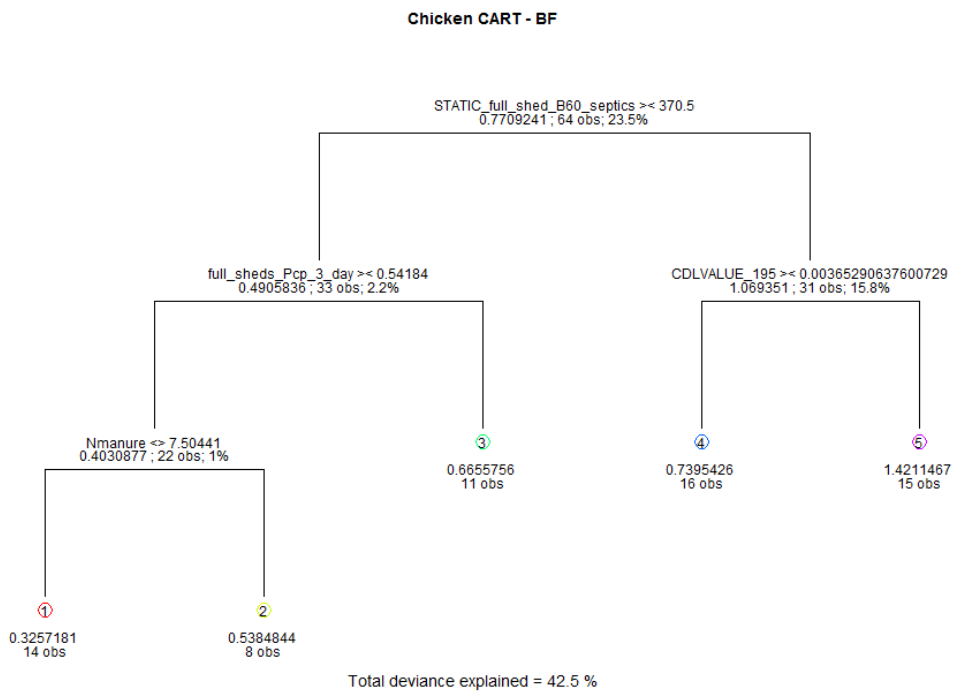
We used Classification and Regression Tree (CART) analysis to compare nutrients and sources tracking results to other chemical, hydrological, and physical, environmental, and land use variables (Martin et al. 2011). CART is a statistical method that splits the dependent variable (nutrients and MST markers) into homogeneous categories based on the influence of independent variables (landscape, agricultural, and climatic characteristics).

## APPENDIX B CART Analysis (Dr. Sherry Martin)

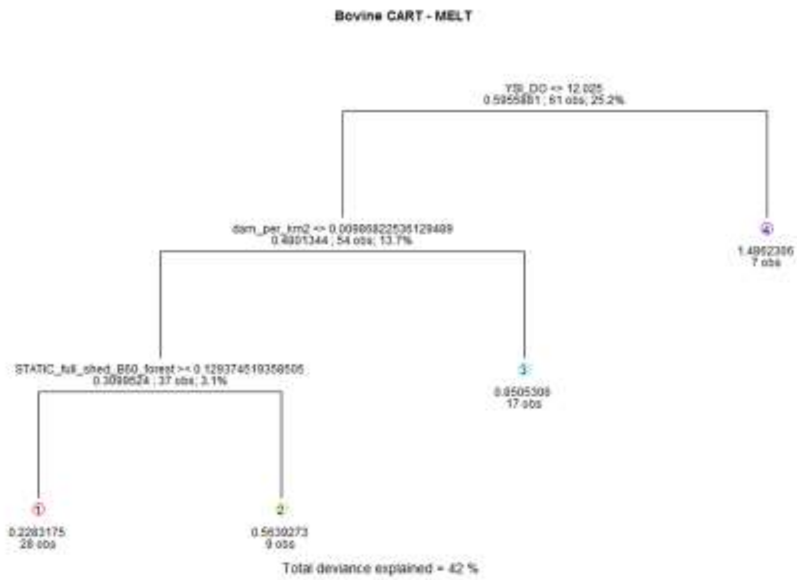
a) Baseflow

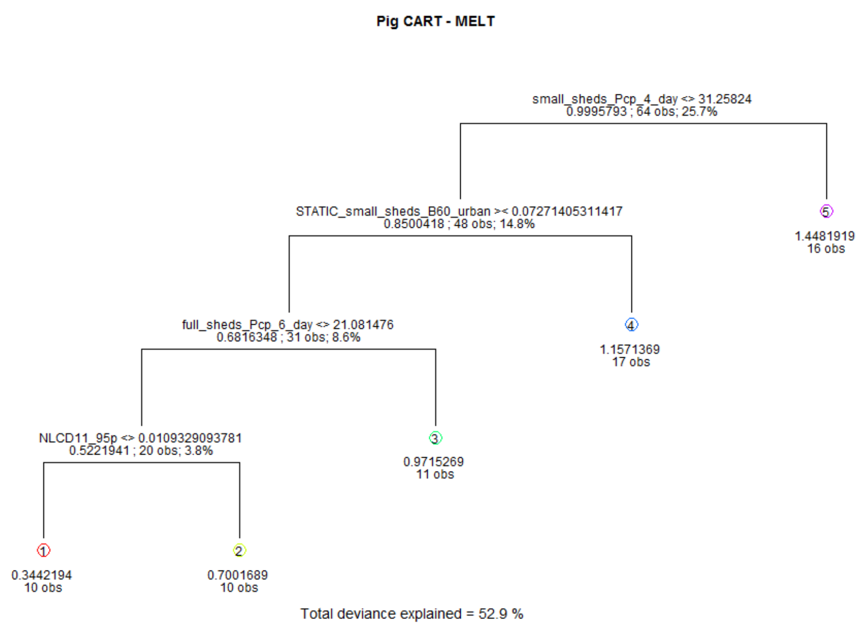


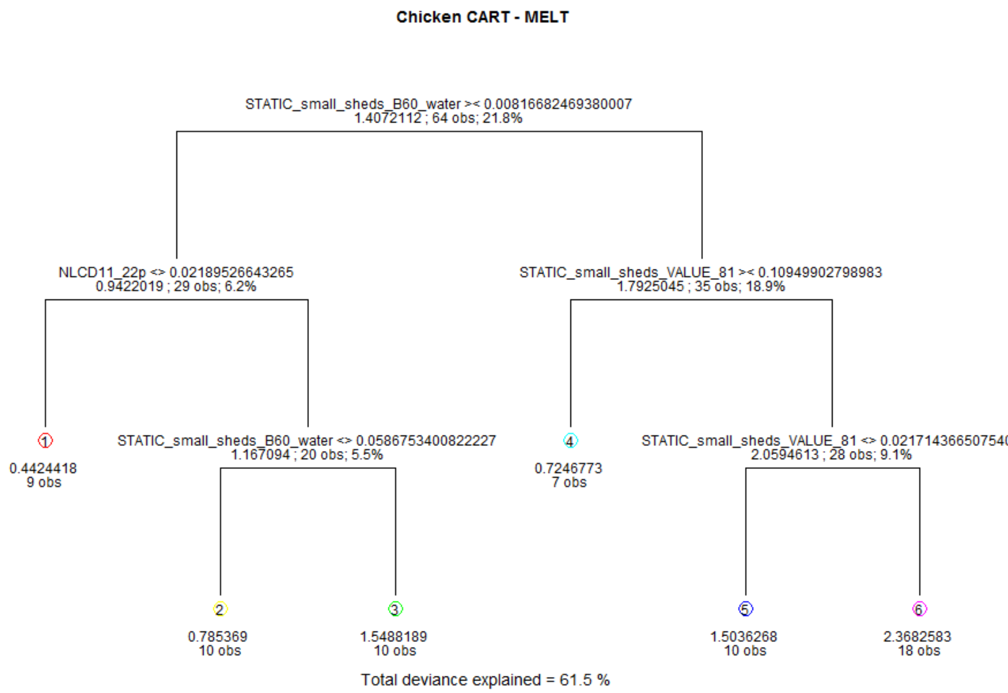




Chicken CART - BF
STATIC_full_shed_B60_septics >< 370.5
0.7709241 ; 64 obs; 23.5%
full_sheds_Pcp_3_day >< 0.54184
0.4905836 ; 33 obs; 2.2%
CDLVALUE_195 >< 0.00365290637600729
1.069351 ; 31 obs; 15.8%
Nmanure <> 7.50441
0.4030877 ; 22 obs; 1%
3
0.6655756
11 obs
4
0.7395426
16 obs
5
1.4211467
15 obs
1
0.3257181
14 obs
2
0.5384844
8 obs
Total deviance explained = 42.5 %

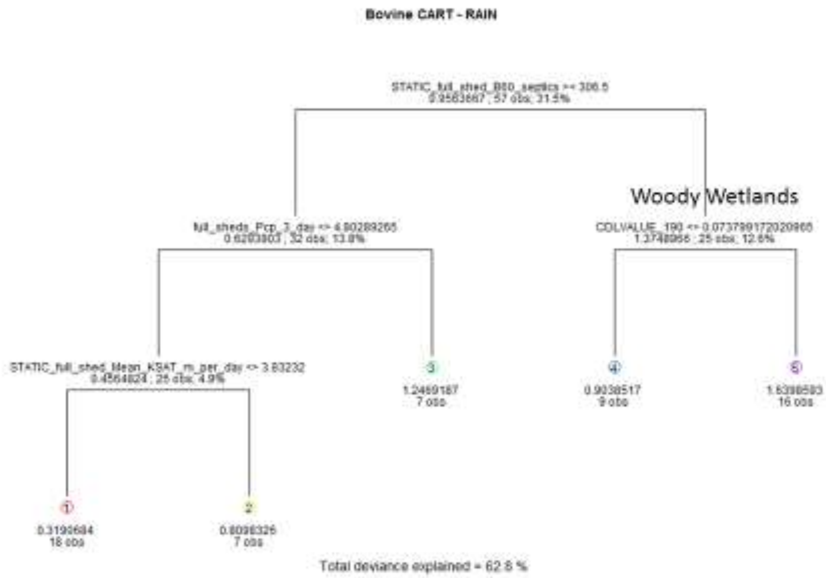
Spring Melt

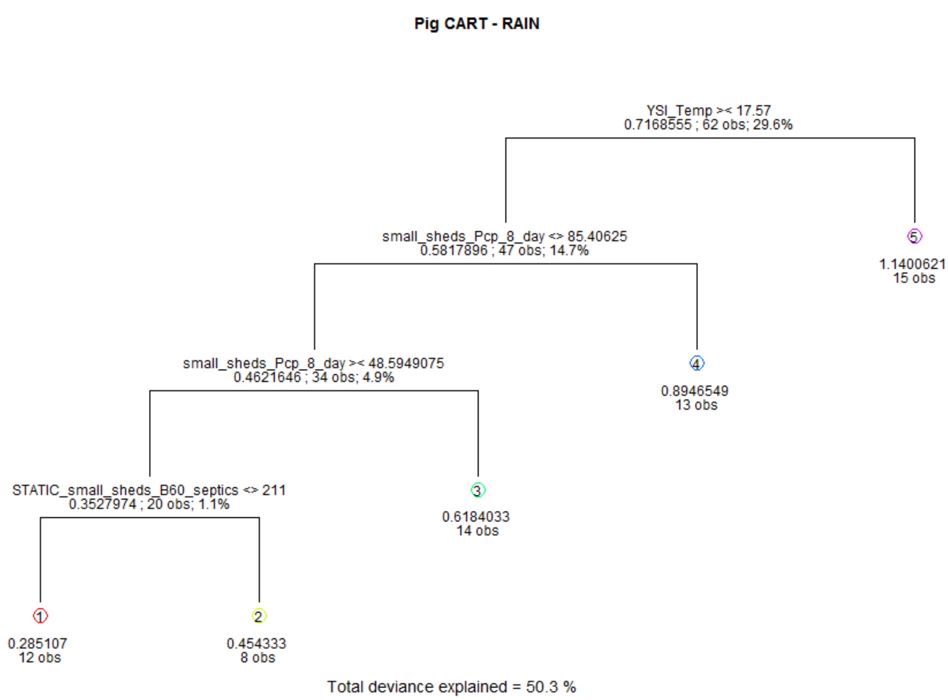

Bovine CART - MELT
YSI_DO <> 12.025
dam_per_km2 <>
STATIC_full_shed_B60_forest >< 0.129374519358505
0.3099624 ; 37 obs; 3.1%
1.4862306
7 obs
0.8505308
17 obs
0.2283175
28 obs
0.5639273
9 obs
Total deviance explained = 42 %

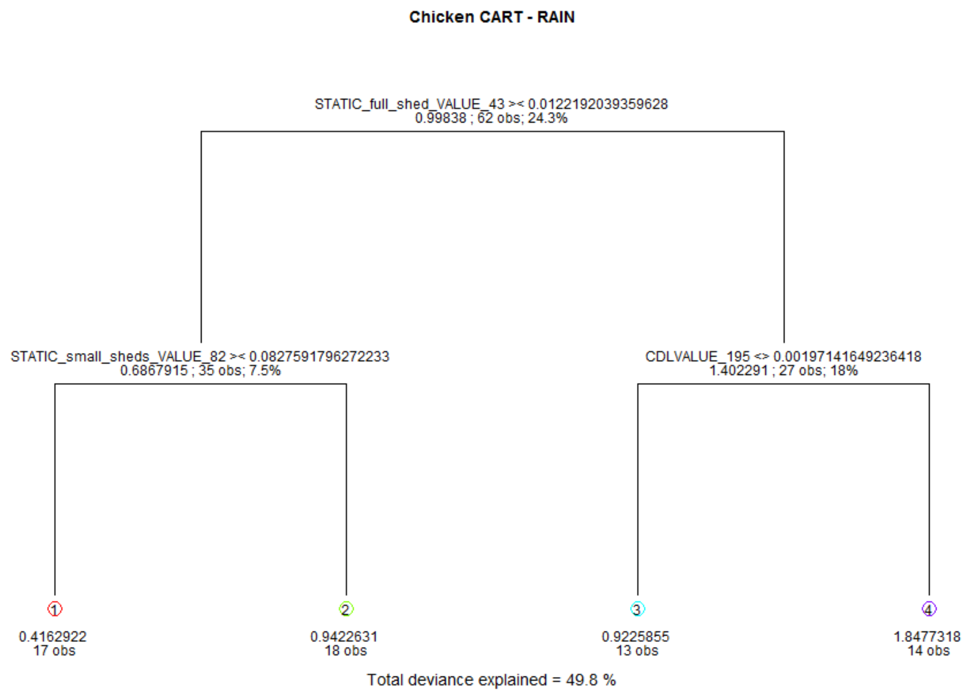

Pig CART - MELT
small_sheds_Pcp_4_day <> 31.25824
0.9995793 ; 64 obs; 25.7%
STATIC_small_sheds_B60_urban >< 0.07271405311417
0.8500418 ; 48 obs; 14.8%
5
1.4481919
16 obs
full_sheds_Pcp_6_day <> 21.081476
0.6816348 ; 31 obs; 8.6%
4
1.1571369
17 obs
NLCD11_95p <> 0.0109329093781
0.5221941 ; 20 obs; 3.8%
3
0.9715269
11 obs
1
0.3442194
10 obs
2
0.7001689
10 obs
Total deviance explained = 52.9 %


Chicken CART - MELT
STATIC_small_sheds_B60_water >< 0.00816682469380007
1.4072112 ; 64 obs; 21.8%
NLCD11_22p <> 0.02189526643265
0.9422019 ; 29 obs; 6.2%
STATIC_small_sheds_VALUE_81 >< 0.10949902798983
1.7925045 ; 35 obs; 18.9%
1
0.4424418
9 obs
STATIC_small_sheds_B60_water <> 0.0586753400822227
1.167094 ; 20 obs; 5.5%
4
0.7246773
7 obs
STATIC_small_sheds_VALUE_81 <> 0.02171436650754(
2.0594613 ; 28 obs; 9.1%
2
0.785369
10 obs
3
1.5488189
10 obs
5
1.5036268
10 obs
6
2.3682583
18 obs
Total deviance explained = 61.5 %

C) Summer Rain

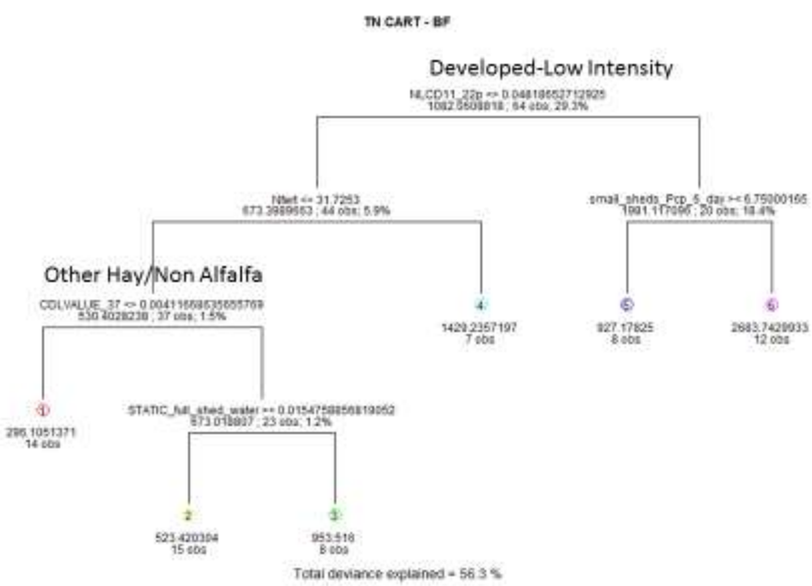


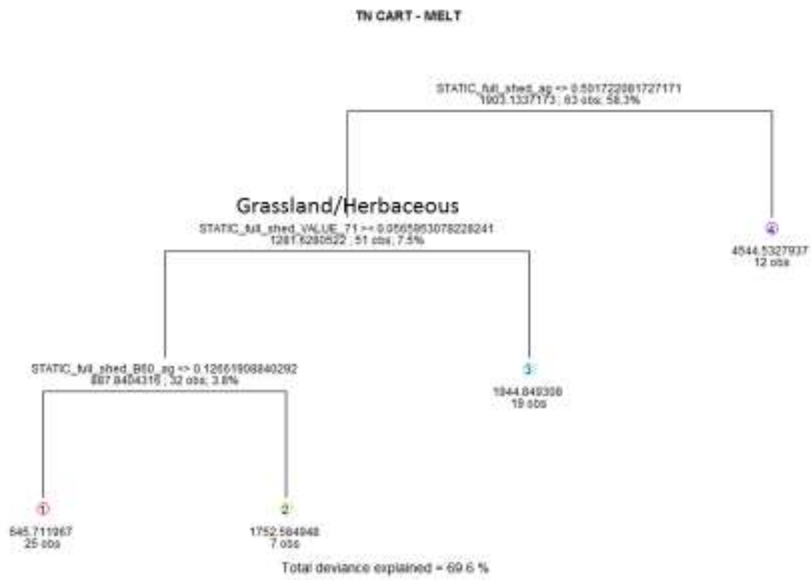



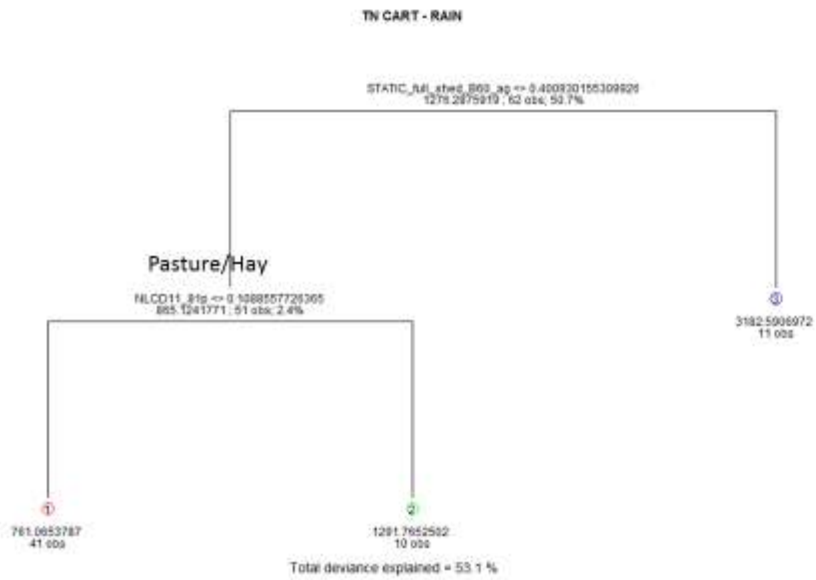

Chicken CART - RAIN
STATIC_full_shed_VALUE_43 >< 0.0122192039359628
0.99838 ; 62 obs; 24.3%
STATIC_small_sheds_VALUE_82 >< 0.0827591796272233
0.6867915 ; 35 obs; 7.5%
CDLVALUE_195 <> 0.00197141649236418
1.402291 ; 27 obs; 18%
1
0.4162922
17 obs
2
0.9422631
18 obs
3
0.9225855
13 obs
4
1.8477318
14 obs
Total deviance explained = 49.8 %

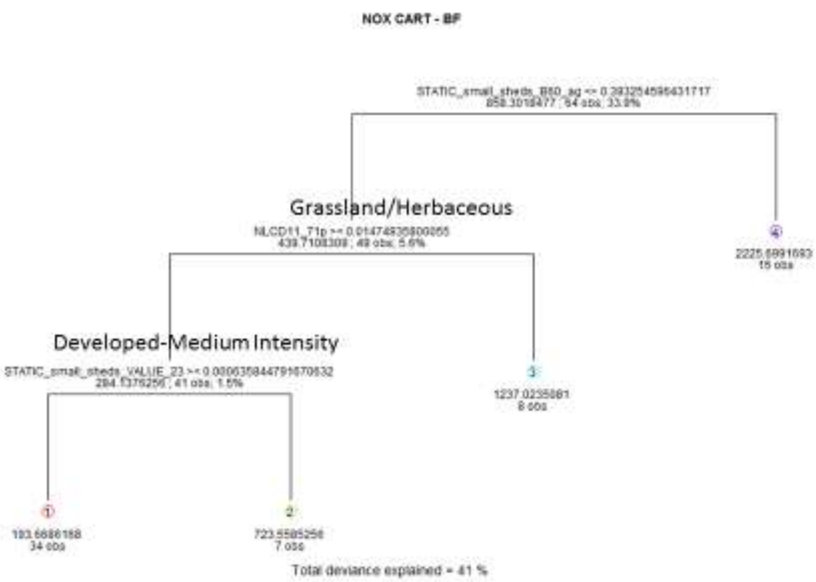
# Nutrient CARTs

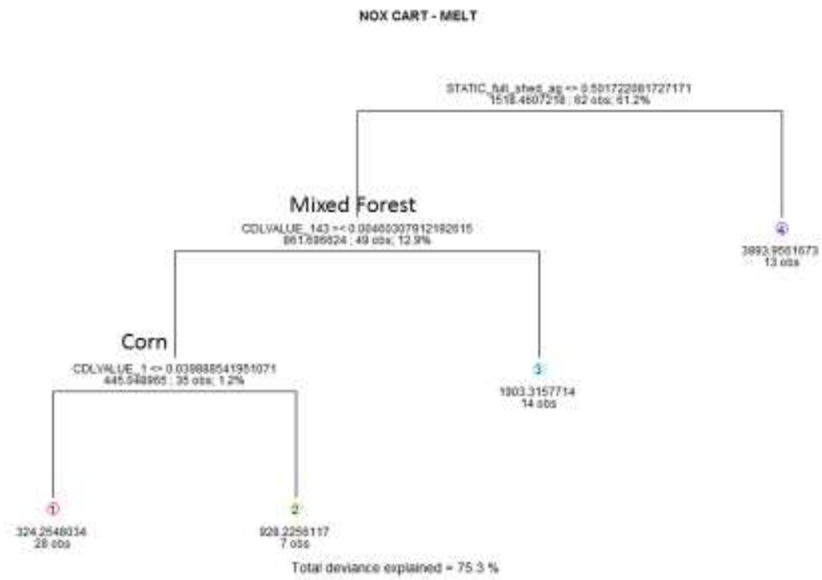
- CART calls included variables from MST CARTs (above) –*plus*– MST markers specific to each season

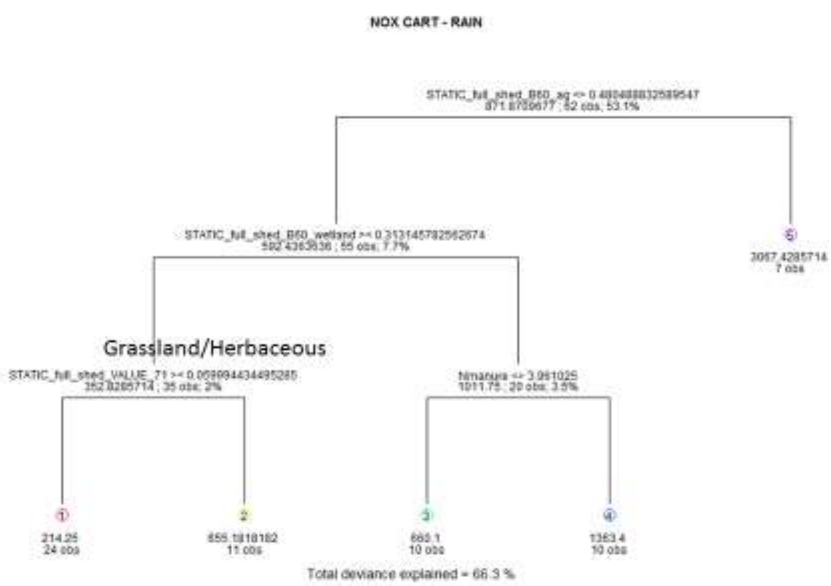


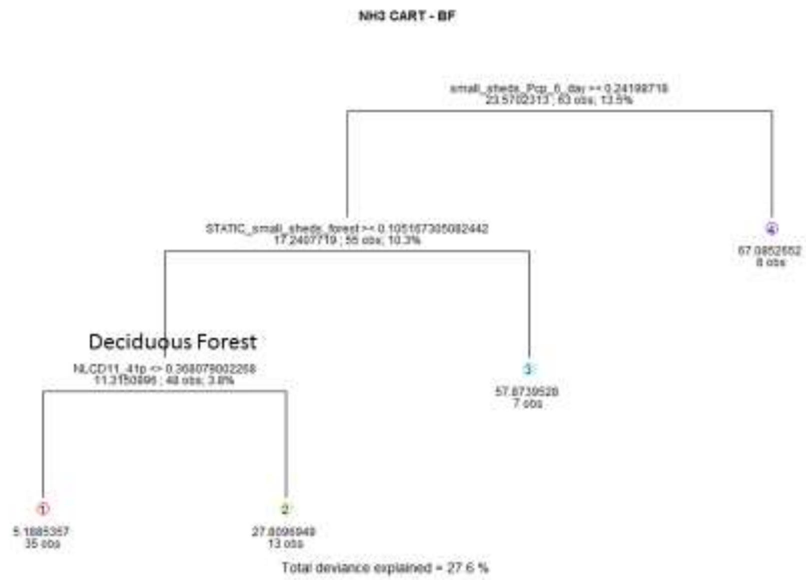

TN CART - MELT
Grassland/Herbaceous
12 obs
19 obs
25 obs
7 obs
Total deviance explained = 69.6 %

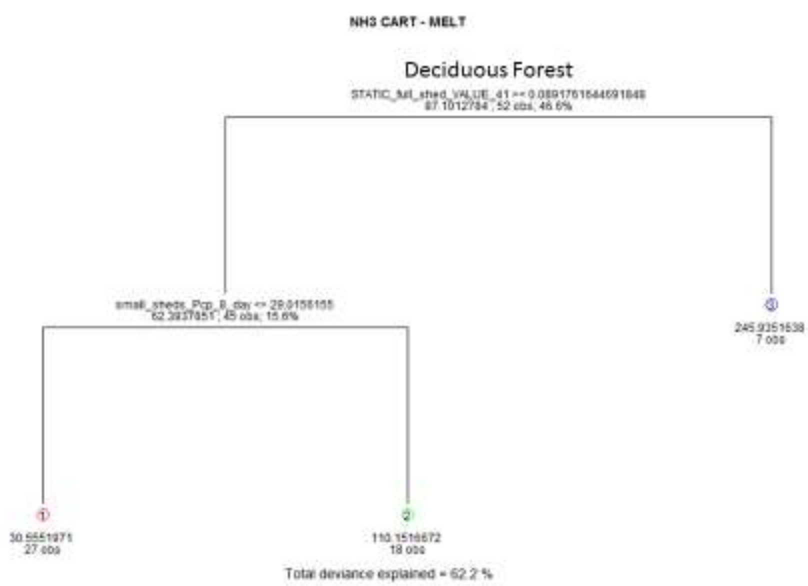

TN CART - RAIN
Pasture/Hay
3182.5906972
11 obs
761.0653787
41 obs
1291.7652502
10 obs
Total deviance explained = 53.1 %

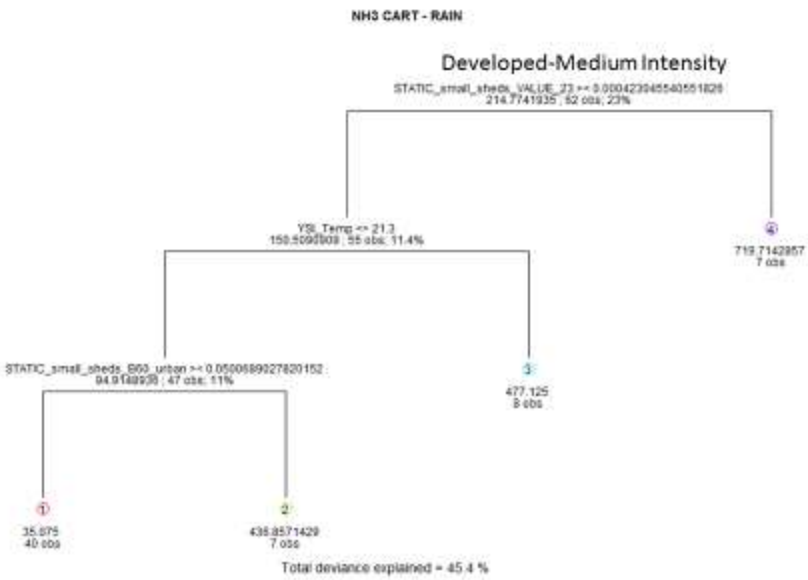

NOX CART - BF
Grassland/Herbaceous
2225.6891693
15 obs
Developed-Medium Intensity
1237.0235081
8 obs
7 obs
34 obs
Total deviance explained = 41 %

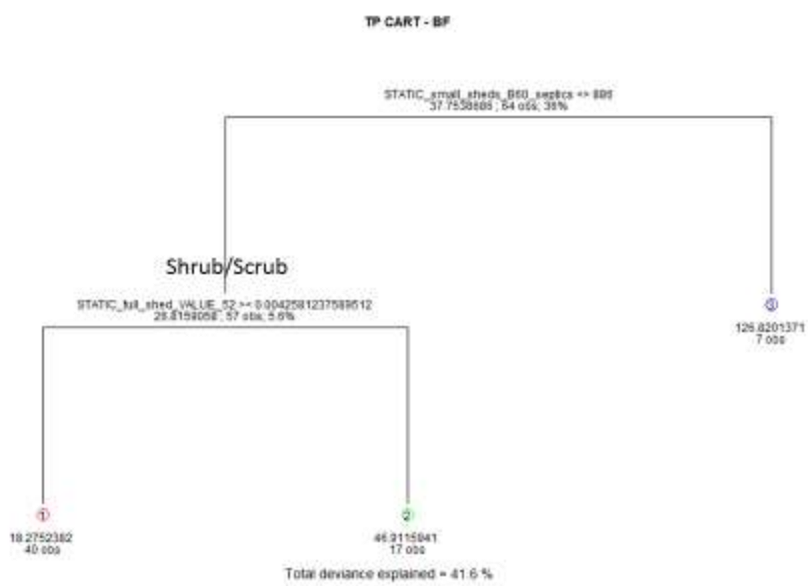

NOX CART - MELT
Mixed Forest
Corn
324.2548034
28 obs
928.2256117
7 obs
1903.3157714
14 obs
3893.9661673
13 obs
Total deviance explained = 75.3 %

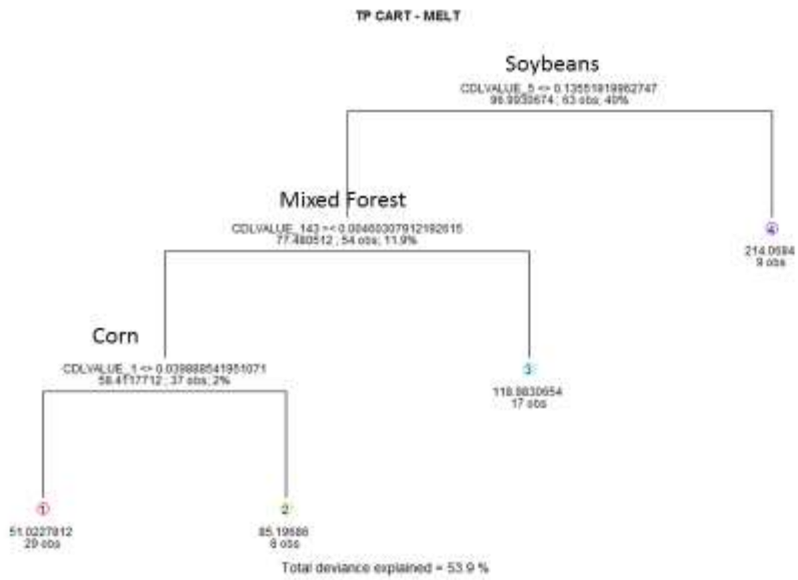

NOX CART - RAIN
Grassland/Herbaceous
214.25
24 obs
655.1818182
11 obs
10 obs
1363.4
10 obs
7 obs
Total deviance explained = 66.3 %

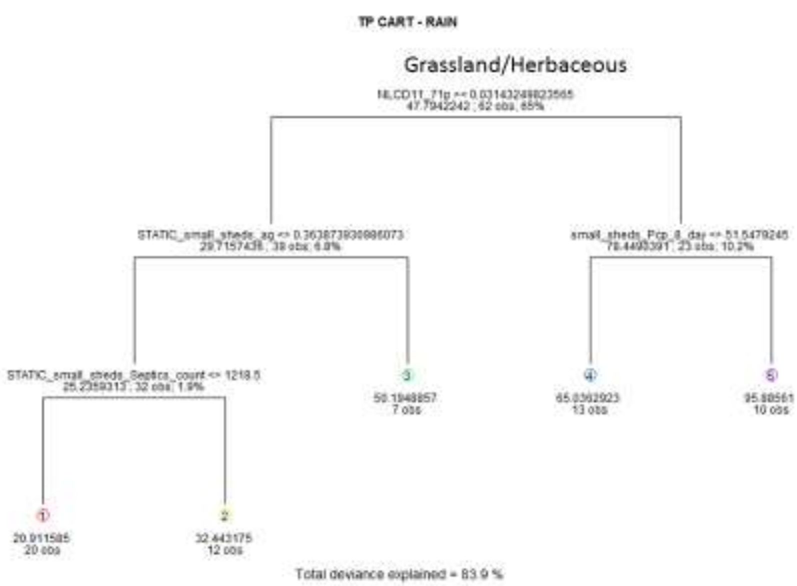

NH3 CART - BF
Deciduous Forest
5.1885357
35 obs
27.8096949
13 obs
57.8739528
7 obs
67.0852552
8 obs
Total deviance explained = 27.6 %

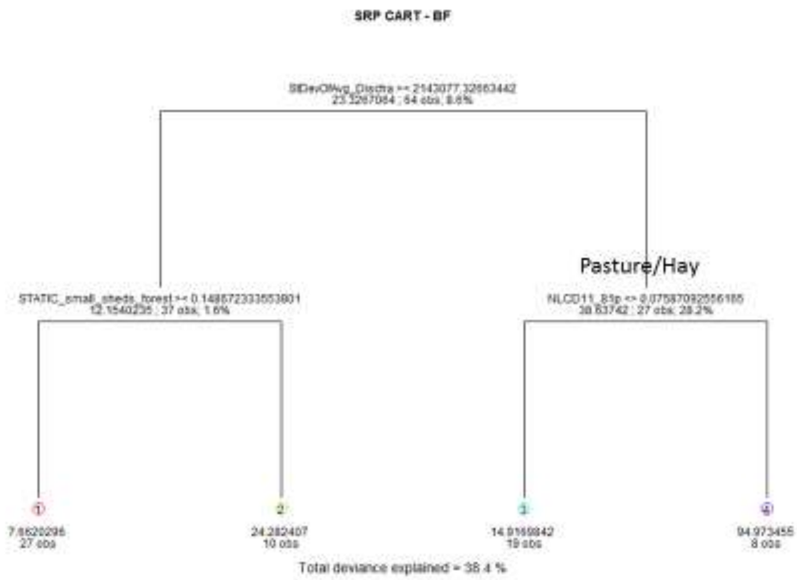

NH3 CART - MELT
Deciduous Forest
30.5551971
27 obs
110.1516672
18 obs
245.9351538
7 obs
Total deviance explained = 62.2 %

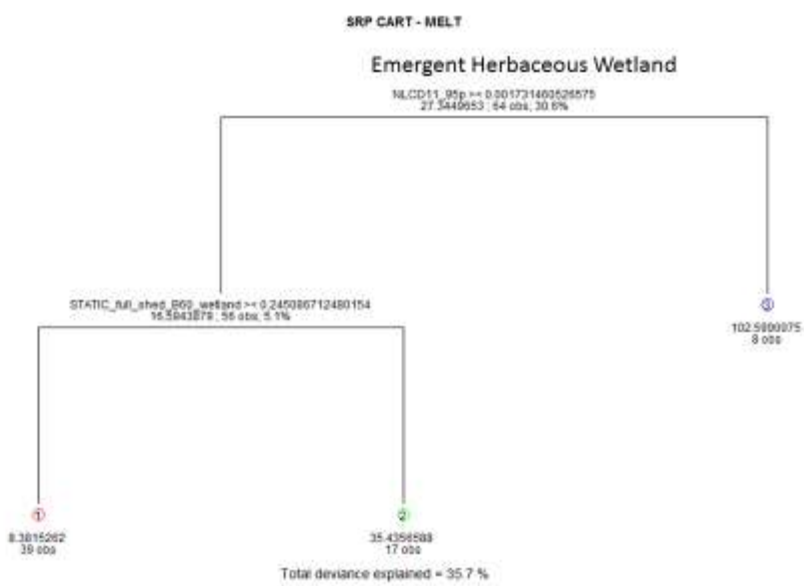

NH3 CART - RAIN
Developed-Medium Intensity
YSI_Temp <= 21.3
719.7142857
7 obs
477.125
8 obs
35.875
40 obs
436.8571429
7 obs
Total deviance explained = 45.4 %

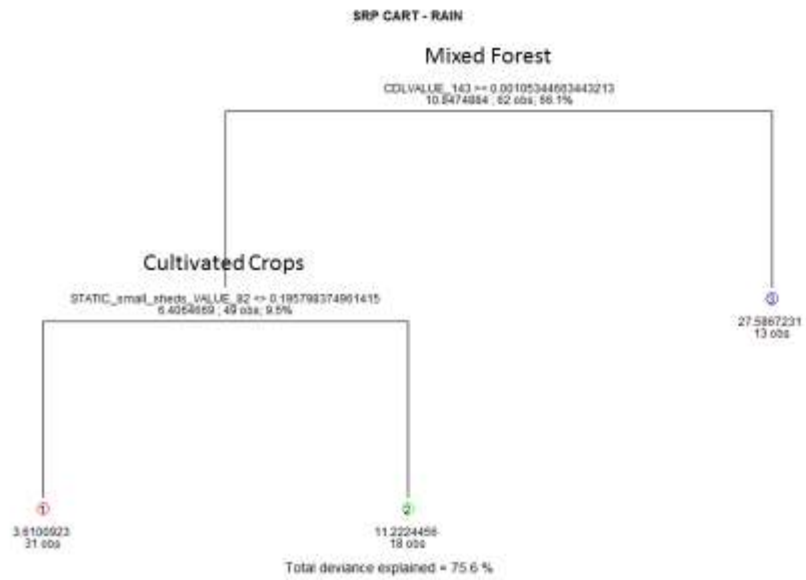

TP CART - BF
Shrub/Scrub
126.8201371
7 obs
18.2752382
40 obs
46.9115941
17 obs
Total deviance explained = 41.6 %


TP CART - MELT
Soybeans
CDLVALUE_5 <= 0.13551919962747
99.9933674 ; 63 obs; 40%
Mixed Forest
CDLVALUE_143 >< 0.00460307912192615
77.480512 ; 54 obs; 11.9%
Corn
CDLVALUE_1 <= 0.039888541951071
58.4117712 ; 37 obs; 2%
1
51.0227812
29 obs
2
85.19686
8 obs
3
118.8830654
17 obs
4
214.0684
9 obs
Total deviance explained = 53.9 %


TP CART - RAIN
Grassland/Herbaceous
NLCD11_71p >= 0.03143249823565
47.7942242 ; 62 obs; 65%
STATIC_small_sheds_ag <= 0.363873930986073
29.7157436 ; 39 obs; 6.8%
small_sheds_Pcp_8_day <= 51.5479245
78.4490391 ; 23 obs; 10.2%
STATIC_small_sheds_Septics_count <= 1218.5
25.2359313 ; 32 obs; 1.9%
1
20.911585
20 obs
2
32.443175
12 obs
3
50.1948857
7 obs
4
65.0362923
13 obs
5
95.88561
10 obs
Total deviance explained = 83.9 %


SRP CART - BF
Pasture/Hay
7.6620296
27 obs
24.282407
10 obs
14.9169842
19 obs
94.973455
8 obs
Total deviance explained = 38.4 %


SRP CART - MELT
Emergent Herbaceous Wetland
102.5900075
8 obs
8.3815262
39 obs
35.4356588
17 obs
Total deviance explained = 35.7 %


SRP CART - RAIN
Mixed Forest
Cultivated Crops
27.5867231
13 obs
3.6100923
31 obs
11.2224456
18 obs
Total deviance explained = 75.6 %

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