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Advancing Understanding of Land Use and Physicochemical Impacts on Fecal Contamination in Mixed-Land-Use Watersheds

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Abstract: Understanding mixed-land-use practices and physicochemical influences on *Escherichia (E.) coli* concentrations is necessary to improve water quality management and human health. Weekly stream water samples and physicochemical data were collected from 22 stream gauging sites representing varying land use practices in a contemporary Appalachian watershed of the eastern USA. Over the period of one annual year, *Escherichia (E.) coli* colony forming units (CFU) per 100 mL were compared to physicochemical parameters and land use practices. Annual average *E. coli* concentration increased by approximately 112% from acid mine drainage (AMD) impacted headwaters to the lower reaches of the watershed (approximate averages of 177 CFU per 100 mL vs. 376 CFU per 100 mL, respectively). Significant Spearman's correlations ($p < 0.05$) were identified from analyses of pH and *E. coli* concentration data representing 77% of sample sites; thus highlighting legacy effects of historic mining (AMD) on microbial water quality. A tipping point of 25–30% mixed development was identified as leading to significant ($p < 0.05$) negative correlations between chloride and *E. coli* concentrations. Study results advance understanding of land use and physicochemical impacts on fecal contamination in mixed-land-use watersheds, aiding in the implementation of effective water quality management practices and policies.

Keywords: *Escherichia coli*; physicochemistry; water quality; land use practices; experimental watershed

1. Introduction

Fecal microbes (e.g., *Escherichia (E.) coli*) are sources of waterborne pathogens and water contamination, causing substantial mortality and morbidity among human populations globally [1,2]. Outbreaks of diarrhea, urinary tract infections, respiratory illness, and pneumonia have been traced to increased fecal microbes (e.g., *E. coli*) in freshwater systems [3,4]. In 2018, the World Health Organization (WHO) reported that waterborne diarrheal diseases are the leading cause of mortality in the developing world, causing 2.2 million human deaths annually [5]. Obviously, understanding factors conducive to elevated fecal microbe (e.g., *E. coli*) concentrations in receiving waters is important from water quality and human health perspective. Improved understanding of factors favorable to fecal microbes can be used to inform land use managers in terms of how to effectively reduce fecal contamination, thereby improving water quality, fresh water security, and human health [6].

Fecal microbes in receiving waters can be variously impacted by physicochemical parameters. Previous investigations reported negative correlations between temperature, salinity, oxygen content, pH, and fecal microbe concentrations [7]. However, information on fecal microbes and physicochemical parameters in freshwater systems is limited as most previous investigations occurred in saline environments [7,8]. The available freshwater investigations comprised notable shortcomings including: short sampling periods and/or few collected samples [9], limited sampling locations (in similar land use types) [9], long periods of time (weeks or months) between sample collection [10], sample collection solely during baseflow conditions [10], or failure to account for constituents such as chloride (Cl^-), which has been shown to influence microbe concentrations in laboratory settings [10,11]. Studies from freshwater systems affirmed relationships between temperature, pH, and fecal microbe concentrations [9,10,12]. However, investigations, including many physicochemical (e.g., chloride) relationships with fecal contamination, are greatly lacking. There is, therefore, need for investigations that include high spatio-temporal sampling regimes, occurring at different stage (streamflow and hydro-climate) conditions, in areas comprising different land use practices, over longer time periods. Information gained from these investigations could aid in the implementation of effective strategies to reduce fecal microbe concentrations in receiving waters.

Land use practice impacts on fecal contamination is relatively well-documented [6,13–18]. Both agricultural and urban land use practices have been shown to generally increase fecal concentrations [6,17], attributed to livestock husbandry [14], manure application [16], or poorly maintained wastewater infrastructure [17], and increased impervious surfaces [19,20]. Previous work reported negative correlations between forested areas and fecal concentrations in receiving waters [21]. Shortcomings of previous work include that many investigations occurred in areas comprising similar land use practices (i.e., lacking variability) [22,23] or included few sampling locations [24]. Therefore, information regarding the influence of land use practices on fecal microbe concentrations in mixed-land-use watersheds (comprising the majority of watersheds globally) are quite limited. Furthermore, the predominant focus on storm events [25], limits information regarding the influence of land use practices on *E. coli* concentrations during other flow and transport conditions. Simultaneous combined analysis of fecal pollution (*E. coli* concentration), land use practices, and physicochemical parameters relationships is also lacking in previous investigations. Such advanced integrated understanding will provide land use managers with more detailed information regarding land use impacts on fecal pollution; thereby, aiding in freshwater quality management decisions.

Multiple study designs and sampling regimes have been implemented to investigate fecal pollution in receiving waters, including laboratory and field based designs. Laboratory studies usually included simulations [26], whereas field based designs comprise event based sampling [27], periodic sampling [19], stochastic sampling [28] and, in limited number, scale-nested experimental watersheds [6]. The scale-nested experimental watershed study design has been particularly effective for quantifying factors (e.g., physicochemical parameters, land use practices) influencing response variables of interest (e.g., *E. coli* concentration) in receiving waters, particularly in mixed-land-use watersheds [29–36]. To achieve this, nested watershed study designs divide larger watersheds into a series of sub-catchments, each with a monitoring (gauging) site at its drainage terminus [30,32,36–38]. Hydrologic characteristics and land us practices can then be isolated though sub-catchment delineation [36]. Quantification of the influencing processes recorded at the sub-catchment scale allows for the identification of the influence and cumulative effect of various land use practices on the response variable of interest [39]. Given its use in numerous peer reviewed publications over multiple decades, the scale-nested and paired [36]) experimental watershed study design is the optimal study design for investigating current knowledge gaps regarding fecal contamination, physicochemical parameters, and land use practices.

The widespread, frequent, and persistent fecal pollution characteristics of the Appalachian region of the USA are similar (representative) to numerous locations globally. The region is, therefore, well suited for (transferrable) research into factors affecting fecal contamination in receiving waters [40]. Water quality and security is a primary concern in rural Appalachia, as fecal contamination poses substantial risk to residents [41]. The risk is elevated by inadequate wastewater treatment infrastructure, geographical isolation, inaccessible terrain, and poverty [41]. Fecal contamination is, therefore, a primary concern, and improved understanding of factors increasing fecal contamination can inform better management practices and improve water quality in the region. Furthermore, the Appalachian region is also physiographically diverse, consisting of distinct geographic, climatological, and ecological areas, typically divided into distinct Northern, Central and Southern regions [42]. The diverse physiography makes results from investigations in these distinct regions comparable and transferable to similar areas globally. For example, the central Appalachian region is comparable to areas such as Hokkaido or Northern Honshu in Japan as these areas comprise temperate climates and well-distributed year-round rainfall [43,44].

The overarching objective of the current work was to quantitatively compare relationships between fecal concentration *(E. coli* colony forming units), physicochemical parameters, and land use practices. Sub-objectives included (1) identifying the dominant factors that influence fecal microbe concentrations in receiving waters, and (2) investigating the effects of seasonal variation on factors (e.g., physicochemical parameters, land use practices) influencing fecal microbes (*E. coli*) in freshwater streams. Study outcomes provide new quantitative insight to these issues thereby providing land use managers with advanced science-based information to improve management practices and policies in freshwater systems.

2. Materials and Methods

2.1. Study Site Description

The location for the current work was a 3rd order tributary of the Monongahela River, the 23 km^2 mixed–land use urbanizing West Run Watershed (WRW). The WRW is located in Morgantown, WV, USA, and contains many different land use practices ranging from various mixed development (e.g., urban and commercial), agriculture, and forested practices. Based on 2016 National Agriculture Imagery Program (NAIP) land use and land cover data, at the time of this investigation WRW consisted of 42.7% forested, 37.7% mixed development and 19.4% agricultural land use (Table 1, site #22). West Run Creek, the primary drainage of WRW includes small floodplains and is a narrow, moderately entrenched stream [6,45]. The elevation in WRW ranges between 420 to 240 m above mean sea level from the headwaters to the confluence of the Monongahela River [6]. The geology of WRW comprises numerous Paleozoic era rock outcroppings and the Monongahela series located in the headwaters [6]. Historic mining of the two coal seams in WRW, the Upper Kittanning, and more specifically, the Pittsburg coal seam resulted in pervasive water quality problems in the watershed (particularly in the headwaters) [6,46].

The climate regime in West Virginia and the city of Morgantown, residing in part, in the WRW and in Monongalia County WV, has a climate lacking a dry season and warm summers (average monthly temperature > 22 °C) and cold winters (average monthly temperature < 0 °C) [47]. Between 1981 and 2010 Morgantown received approximately 1060 mm of average annual precipitation, with the wettest and warmest month (July) comprising an average daily temperature and monthly precipitation of 23 °C and 117 mm, respectively [48]. Conversely, the coldest (January) month included an average daily temperature of −0.4 °C and the driest (February) month an average monthly precipitation of 66 mm [48].

A twenty-two study site (i.e., n = 22 stream gauging sites) scale-nested and paired experimental watershed study design [30,39,49–51] was implemented in WRW in 2016. Field surveys and GIS were used to identify study site locations and associated sub-catchments. The sampling sites (numbered in downstream order were located on the 1st and 2nd order confluence tributaries of WRW (#1, #2, #5, #7, #8, #9, # 11, #12, #14, #15, #16, #17, and #20) and along West Run Creek (#3, #4, #6, #10, #13, #18, #19, #21, and #22) and included many land use practices (Table 1; Figure 1). Forested land use was the predominant land use practice in WRW during the time of the investigation, accounting for 42.7% of the total land use practices in the watershed (Table 1, site #22). All the sub-catchments except #1, #11,

#15, #16, and #20 were by majority forested. Sub-catchments #11 and #16 were primarily agricultural, whereas sub-catchments #1, #15 and #20 were primarily mixed development (Table 1).

Table 1. Land use/land cover characteristics (% cover) of 22 monitoring sites in West Run Watershed (WRW), West Virginia, USA, including total drainage area (km^2).

Site	Mixed Development (%)	Agriculture (%)	Forested (%)	Drainage Area (km^2)
1	53.23%	38.70%	8.07%	0.30
2	13.58%	12.20%	74.21%	0.29
3	22.35%	16.17%	61.32%	1.87
4	25.88%	14.91%	59.00%	2.48
5	23.35%	25.51%	51.14%	0.38
6	23.91%	17.25%	58.70%	3.72
7	16.33%	28.60%	54.91%	0.78
8	30.78%	16.47%	52.35%	1.55
9	27.57%	19.33%	52.84%	2.29
10	24.92%	18.40%	56.49%	6.18
11	18.15%	41.87%	39.16%	1.75
12	31.77%	33.72%	34.51%	1.75
13	26.83%	25.77%	47.15%	10.53
14	16.19%	26.43%	56.92%	3.36
15	70.28%	10.31%	19.42%	0.98
16	5.38%	58.72%	35.16%	0.25
17	4.78%	9.38%	85.84%	0.75
18	25.98%	24.88%	48.86%	16.41
19	29.45%	22.45%	47.85%	18.88
20	89.16%	4.19%	6.61%	3.42
21	38.10%	19.46%	42.23%	22.93
22	37.71%	19.38%	42.66%	23.24

Note: due to the omission of certain categories (i.e., wetland, open water, etc.) and certain categories comprising combinations other others (e.g., mixed development = urban and residential) land use percentages may not sum to 100%. Final row (site #22) indicates total values for the entire watershed.

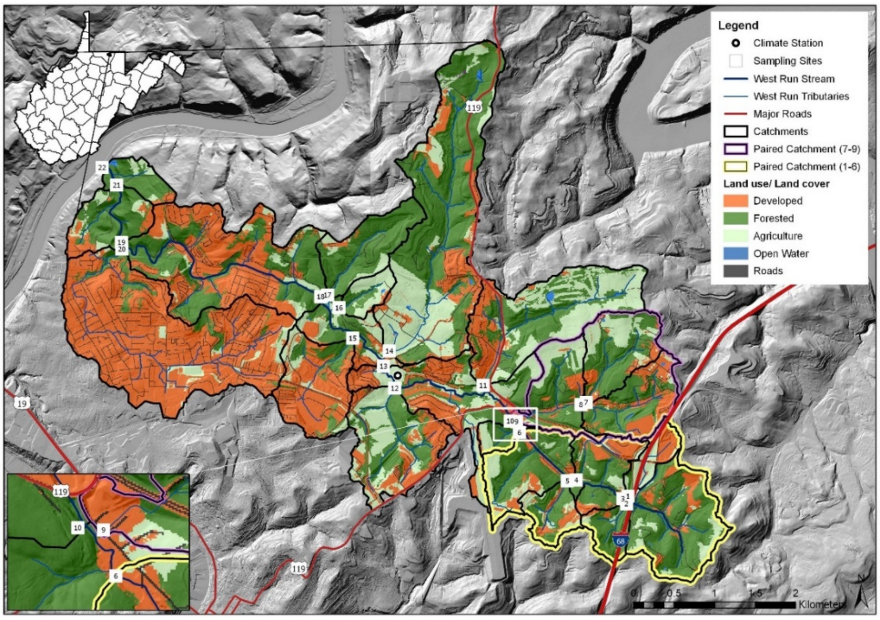

Figure 1. Land use/ land cover of West Run Watershed, Morgantown, WV, USA, including monitoring/ sampling locations for the current investigation.

The predominance of forested cover among the sites should not be taken to imply that catchments were equivalent in terms of land use. For example, the reference sub-catchment (control) for the current work, sub-catchment #17 comprised 85.84% forested land use 9.4% agricultural and 4.8% mixed development land use practices. This sub-catchment comprised the largest percentage of forested land use practices among the sub-catchments (hence, selection as reference sub-catchment). Conversely, sub-catchment #12 comprised 34.5% forested, 33.7% agriculture, and 31.7% agriculture land use practices and had the lowest percentage forested land use among the primarily forested sites (Table 1). Sub-catchment #17 is therefore distinct in terms of land use practices relative to sub-catchment #12, despite both being predominantly forested. In general, at the time of the investigation, mixed development comprised the second largest percentage of land use practices (37.7%) and agricultural land use practices accounted for the lowest percentage of land use practices (19.4%) in WRW.

2.2. Data Collection

Climate data, including precipitation (Campbell Scientific TE525 Tipping Bucket Rain Gauge), average air temperature, relative humidity (Campbell Scientific HC2S3 Temperature and Relative Humidity Probe), and average wind speed (Campbell Scientific Met One 034B Wind Set instrument), were recorded at a three-meter height within approximately 30 m of site #13 (Figure 1), for the duration of the study period (2 January 2018–1 January 2019).

For the current work, weekly water grab-samples were collected as per Petersen et al. [6], Hubbart et al. [52], Kellner and Hubbart [38], and Zeiger and Hubbart [37,53] from each monitoring site (stream order ≤ 3). Water sample collection proceeded through numerical order of sites starting at 09:00 at site #1. To reduce overall sampling time (increasing sample representativeness during processing), sites #9 and #10 were sampled before sites #7 and #8, due to their proximity relative to site #6 (Figure 1). The calendar year duration of the sampling period (1 February 2018–1 January 2019) was longer than previous work on fecal contamination, allowing for assessment of seasonal variability of *E. coli* concentration, physicochemical, and hydro-climate data [54,55]. This resulted in a more comprehensive quantification of fecal contamination (*E. coli*) regimes at sub-catchment mixed-land-use scales than offered through most, if not all previous studies in the published literature. A total of 1166 spatio-temporally delineated fecal contamination (*E. coli*) concentration values were obtained during this high-resolution study.

Once collected, water samples were transported to the Interdisciplinary Hydrology Laboratory, located in the Davis College of Agriculture, Natural Resources and Design at West Virginia University, for analyses. *Escherichia (E) coli* was used as an indicator organism to quantify fecal contamination as per previous work investigating fecal contamination [2,6]. The Colilert test, developed by IDEXX Laboratories Inc. and approved by the U.S. Environmental Protection Agency (EPA) [56], was used to quantify *E. coli* colony forming units (CFU). The test was designed to eliminate the need for sample dilution when evaluating fecal concentration in water samples and is included in the Standard Methods for Examination of Water and Wastewater [56,57]. The likelihood of reporting inaccurate results (i.e., false positives ± 10%) when using the test is low due to Colilert's Defined Substrate Technology nutrient-indicator (ONPG), and a selectively suppressing formulated matrix. The ONPG is a carbon source that most non-target organisms lack the enzyme to utilize, rendering them unable to grow or interfere [56]. The few non-target organism that can use ONPG as a carbon source is suppressed by a selective matrix [56]. As per Colilert instructions, the Colilert (ONPG) substrate was added to 100 mL of sampled water, sealed in the Quanti-Tray, and incubated at 35 °C for 24 h. The Quanti-Tray system comprises 96 total wells: 48 large wells (49, including an overflow well) and 48 small wells. Results are reported in CFU per 100 mL [56]. Following incubation, fluorescing (positive for *E. coli*) wells were enumerated using a UV light (6 watt, 365 nm wavelength) and compared to the Quanti-Tray Most Probable Number (MPN) table. The MPN table is used to convert the number of positive wells to an *E. coli* concentration value (CFU per 100mL), with a 95% confidence interval. The applied method thus used a MPN approach to estimate *E. coli* CFU concentration; therefore, *E. coli* concentration data was referred to as CFU not MPN during the investigation. The *E. coli* concentration range that could be detected using this method was <1 to 1011.2 CFU. *E. coli* concentrations exceeding 1011 CFU per 100 mL; therefore, could not be estimated accurately. However, this shortcoming was deemed acceptable due to the accuracy at detecting low *E. coli* concentrations provided by the method, which is important when sampling for *E. coli* outside of storm events or in land use areas less prone to fecal contamination.

Concurrent with the collection of water grab samples, five physicochemical variables were collected using a handheld multi-parameter water quality sonde (YSI Inc./Xylem Inc.) fitted with an Ion Selective Electrode (ISE) multi-probe [58]. Variables included water temperature (°C), dissolved oxygen (DO), specific conductance (SPC), pH, and Chloride ion. The ISE probe sensed water temperatures ranging from −5 °C to 70 °C with an accuracy of ±0.2 °C, SPC ranging from 0 to 200 mS cm^{-1} with an accuracy of ±0.5% of reading or ±.001 mS cm^{-1}, whichever is greater, (for readings 0–100 mS cm^{-1}) or ±1.0% of reading (for readings 100–200 mS cm^{-1}), DO ranging from 0 to 50 mg L^{-1}, with an accuracy of ±1% (for readings 0–20 mg L^{-1}) or ±8% (for readings 20–50 mg L^{-1}), pH ranging from 0 to 14 units, with an accuracy of ±0.2 units, and Chloride ranging from 0 to 1000 mg/L (at water temperatures from 0 to 40 °C) with an accuracy ±15% of reading or 5 mg/L (whichever is greater) [58].

2.3. Data Analysis

Descriptive statistics for *E. coli*, physicochemical variables and hydro-climate data aggregated to the study period were calculated. Statistical analyses were conducted using OriginPro 2019 (OriginLab Corporation). The Anderson Darling Test was used for normality testing [59]. Land use practices were reclassified into three major categories prior to analysis including mixed development, agriculture, and forested land use [6]. Mixed development included roads, impervious surfaces, mixed development, and barren areas. Agriculture comprised low vegetation, hay pasture, and cultivated crops. Forested land use constituted mine grass, forest, mixed mesophytic forest, dry mesic oak forest, dry oak (pine) forest, and small stream riparian habitats. Seasonal variation was analyzed by dividing annual data into four quarter data subsets, comprising all weekly samples collected in three-month blocks starting 1 January 2018. Consequently, quarter one included 2 January 2018–27 March 2018 (winter); quarter two included 3 April 2018–26 June 2018 (Spring); quarter three included 3 July 2018–25 September 2018 (summer); and quarter four included 2 October, 2018–1 January, 2019 (fall). Spearman correlation tests for both annual and quarterly datasets, with a significance threshold of $\alpha = 0.05$ [60], were used to analyze the relationship between *E. coli* concentration, physicochemical parameters, and land use practices at all twenty-two sites. Finally, the annual data and quarterly data subsets comprising *E. coli* concentrations, physicochemical parameters, and land use practices were analyzed using principal component analysis (PCA) (presented in biplots) across all 22 sampling locations.

3. Results and Discussion

Given the scope of this research and large subsequent dataset, the authors have included the most salient tables and figures in text to facilitate presentation of results and discussion. For the reader wishing to learn more, comprehensive descriptive statistics tables are provided in Appendix A (referenced throughout).

3.1. Climate during Study

West Run Watershed received approximately 20% more total precipitation in 2018 (1378 mm) than annual averages dating back to 2007 (1096 mm) [61]. October was the driest (47 mm) and September was the wettest (186 mm) months during the study period (Figure 2), with September receiving more than double the historic average precipitation (80 mm) [61]. Consequently, September included approximately 14% of the precipitation received during 2018. During 2018, average annual air temperature (12 °C) was close to the historic annual average (11 °C) [61]. The coldest average monthly temperature in WRW was recorded in January (−4 °C), whereas July had the warmest average monthly temperature (22 °C) [61]. Average annual relative humidity was 76% during 2018 and was, therefore, characteristically high (Figure 2), as is common in the region [61]. Ultimately, the climate in WRW during 2018 was predictably variable and consistent with historic trends. As is characteristic of WRW, and the region, 2018 did not include a dry season; however, quarters two and three (spring and summer) received more precipitation than quarters one and four (winter and fall). This was the result of large precipitation events during quarters two/spring (e.g., 6 May; 24 mm) and three/ summer (e.g., 9 September; 60 mm) (Figure 2) [61].

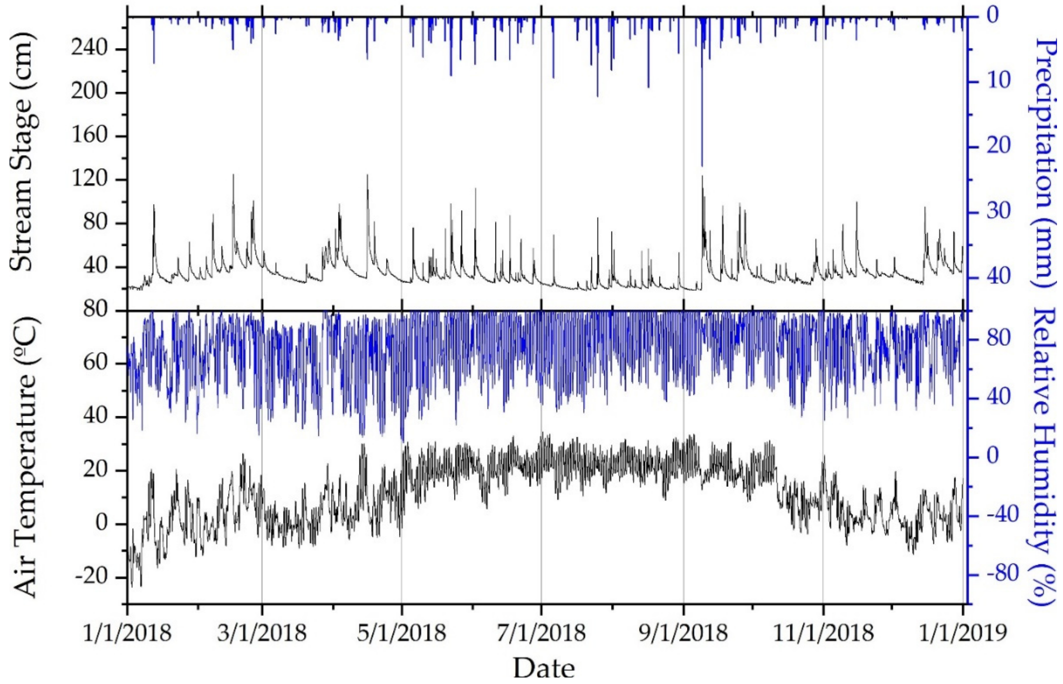

Figure 2. Thirty-minute time series of climate variables during study period (2 January 2018–1 January 2019) in West Run Watershed, West Virginia, USA. Note: Stream stage was monitored in the primary stream of WRW, West Run Creek, at site #13 (Figure 1).

3.2. *E. Coli Concentrations*

Sub-catchment #16, comprising predominantly agricultural land use practices (59%) had the highest *E. coli* concentration (560 CFU per 100 mL) during the current investigation, similar to previous inventions in WRW [6,18]. This sub-catchment also had the highest median concentration (575 CFU per 100 mL) (Figure 3; Appendix A Table A1). These results are supported by previous work that reported significant correlations ($p < 0.04$) between agricultural land use and *E. coli* concentrations [6] and increased fecal contamination in agricultural areas [21,27]. Conversely, forested land use areas (site #2; 74% Forested and site #5; 51% Forested) comprised the lowest *E. coli* concentrations, including the lowest median (3 CFU per 100 mL), and average (34 CFU per 100 mL), respectively (Figure 3; Appendix A Table A1). The low *E. coli* concentrations recorded at these two sites, and others including sites #7, #8, and #9 located in the headwaters of WRW (Figure 1) were impacted by acid mine drainage (AMD), which lowers the pH of receiving waters [62], potentially killing fecal bacteria [63]. For a more comprehensive discussion of pH, the reader is referred to the physicochemical parameters Section 3.3. Under approximately average pH conditions, forested land use areas had lower *E. coli* concentrations (e.g., site # 17: 86% forested average (avg.) 206 CFU per 100 mL) relative to areas comprising either agriculture (e.g., site # 16: 59% agriculture, avg. 560 CFU per 100 mL) or mixed development (e.g., site # 20: 89% mixed development, avg. 415 CFU per 100 mL) (Figure 3). In previous investigations, decreased fecal contamination was recorded in forested areas [21], and attributed to increased receiving water quality [64], thereby supporting the results of the current investigation. The motivation for sampling between storm events, is strengthened by the low average *E. coli* concentrations (specifically in the headwaters) recorded during the study period (2 January 2018—1 January 2019). Results support previous investigations that showed increased *E. coli* concentrations in agricultural areas (e.g., site # 16) and decreased *E. coli* concentrations in forested areas (e.g., site # 17). However, no published investigations included sampling over a full annual year using such a high spatial and temporal resolution. Results here are therefore unique, comprehensive, and may increase confidence in previous study outcomes.

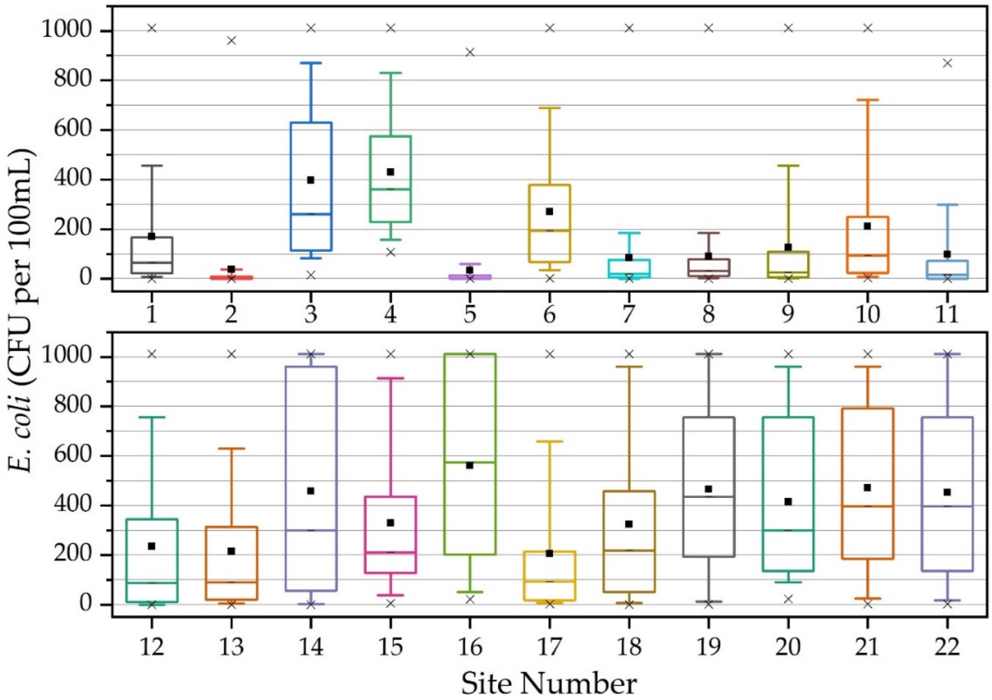

Figure 3. Box and whisker plot of *E. coli* concentration (CFU per 100 mL) descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

3.3. Physicochemical Parameters

Mixed development land use (e.g., site #20: 89% mixed development) had elevated annual average water temperatures (avg. 12.88 °C) relative to agriculture (e.g., site #16: 59% agriculture, avg. 12.00 °C) and forested land use practices (e.g., site #17: 86% forested, avg. 11.67 °C) (Figure 4; Appendix A Table A2). Previous investigations linking elevated receiving water temperature to mixed development areas support the results of the current study [65–68]. Elevated water temperatures in mixed development areas are typically attributed to decreased vegetation (reduced stream shading) and warmer impervious surfaces (e.g., road and building surfaces) and increased surface runoff (volume and temperature) during storm events [65–68]. During the current investigation water temperatures were lower in forested areas (e.g., site #17: 86% forested), including a lower average (11.67 °C), median (10.40 °C), and maximum (21.70 °C) relative to other land uses (Figure 4; Appendix A Table A2), thereby supporting the results from previous work [69]. The study design and high spatial and temporal resolution-sampling regime of the current investigation resulted in an extensive water temperature and *E. coli* concentration dataset. This is important, given temperature has been identified as the primary factor influencing *E. coli* survival in the environment, accounting for up to 61% of the variance (based on inactivation rates) of *E. coli* populations [70]. Therefore, this analysis (see non-parametric results Sections 3.4 and 3.5) expands current understanding of land use practice and water temperature impacts on *E. coli* concentrations.

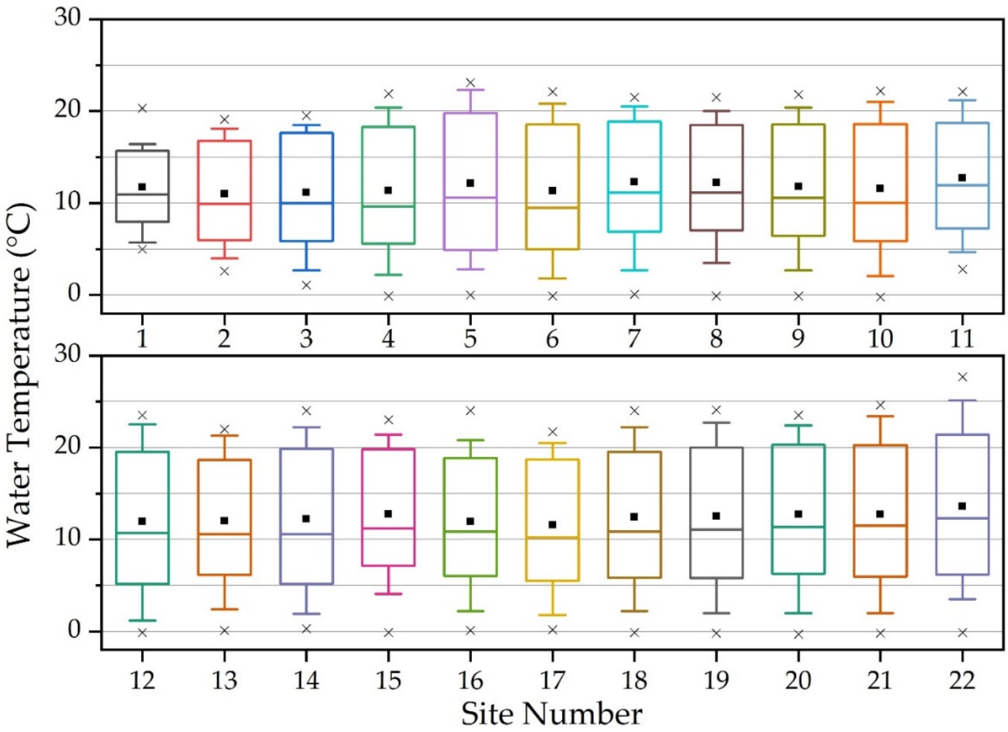

Figure 4. Box and whisker plot of water temperature (°C) descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

Previous work reported decreased pH in WRW, particularly in the headwaters, attributed to historic mining activities and subsequent AMD [6,18,46]. In the current work, approximately 55% of monitoring sites located in the headwaters had average pH values below six (Figure 5; Appendix A Table A3). The lowest pH was recorded in at site #8 with an annual average pH of 4.37 and median of 4.23. Monitoring sites #7, #8, and #9, (comprising one of the paired watersheds) had the lowest pH values among the sites (annual averages 5.03; 4.37; 5.08 and medians 5.04; 4.23; 4.93, respectively). Sampling locations in lower WRW displayed increased pH values, indicating a dilution of the AMD that was prevalent in the headwaters (no historic mining in lower reaches). For example, when comparing the annual average pH of two sites located on West Run Creek (site #13: 6.10 and #18: 7.18) there was a notable increase in pH (1.08) from acidic to neutral (Figure 5; Appendix A Table A3). Ultimately, the pH data recorded during the current investigation provides high sampling density evidence for legacy effects of historic mining practices. Notably, while mining activities in WRW ceased by 1977 [46]. Long-term effects of those practices were still impacting receiving waters at the time of the current study.

Acid mine drainage (AMD) impacted sites, including the paired watershed comprising sites #7, #8 and #9, generally included the highest specific conductance (SPC) data recorded during the study period (2 January 2018–1 January 2019). For example, site #8, which was the most heavily impacted by AMD (see preceding section) comprised the highest average (1661.13 µS/cm) and median (1480.00 µS/cm) SPC values (Figure 6; Appendix A Table A4). These findings are supported by previous work that reported elevated SPC in AMD impacted locations [71], including previous work investigating the influence of coal mining on conductivity of waters in Appalachia [72]. Increased SPC in AMD impacted areas can be attributed to increased iron, sulfate, copper, cadmium, arsenic, and/or other constituents in the water increasing ion availability [73]. Mixed development sub-catchments (sites #15: 70% mixed development and #20: 89% mixed development) also displayed elevated SPC data (averages of 1392.64 µS/cm and 1463.58 µS/cm, respectively). Moreover, these mixed development areas included the highest recorded maximum SPC (6631.00 µS/cm and 6106.00 µS/cm, respectively) (Figure 6; Appendix A Table A4). These results are supported by previous investigations reporting increased SPC in mixed development locations [74,75], attributed to increased ions in receiving water originating from diverse sources, including transportation, sewage treatment, and infrastructure development [75]. Moreover, the low SPC recorded at forested sites (e.g., site #17: 86% forested; average SPC 249.11 µS/cm), in the lower portion of WRW, during the current work is also supported by previous work that reported negative associations between SPC and forested land cover [76].

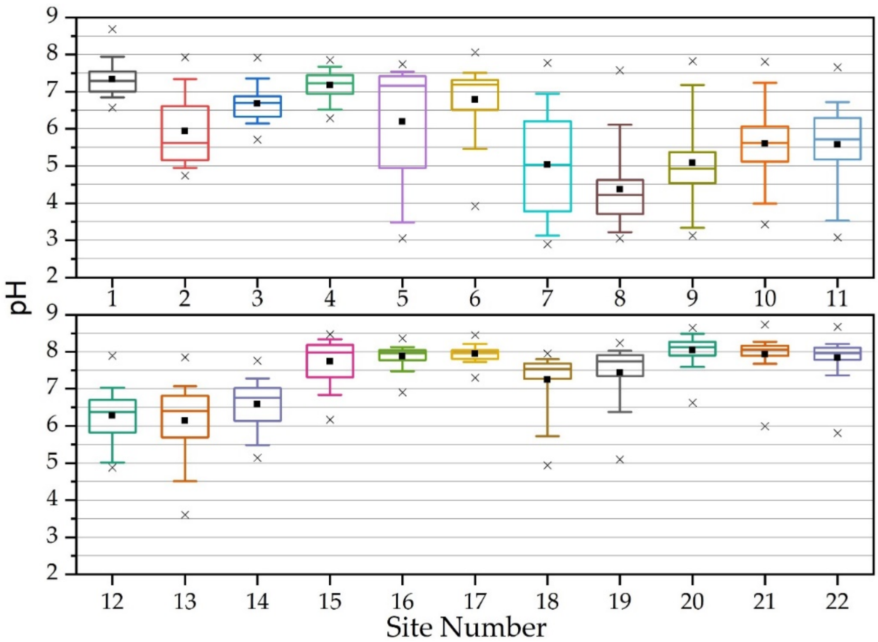

Figure 5. Box and whisker plot of pH descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

Dissolved oxygen (DO) was lowest at site #1, comprising an annual average of 85.93% and median of 85.10%, and the highest at site #20, with an average of 104.63% and median of 102.40% (Figure 7; Appendix A Table A5). Statistical analysis did not reveal a significant (CI = 0.05) relationship between DO and land use practices, attributable to unmeasured DO influencing factors. Variables beyond the scope of the current investigation included ground water depth and antecedent soil water conditions) [77], aquatic macrophytes [78], aquatic plant photosynthesis [79], and aquatic chemical, physical, and biochemical activities [80]. These factors can influence DO independent from changes to land use practices [77–80], thereby, obscuring the influence of land use changes on DO in receiving waters. However, increased DO variability was recorded at mixed development sub-catchments (site #15: 70% mixed development and site #20: 89% mixed development) in the lower portion of WRW (Figure 7). DO in streams can be impacted by urbanization through increased primary production or decomposition of organic matter [81]. Therefore, increased DO variability in mixed development areas can alter microbial community structures [82] of associated receiving waters, potentially indirectly affecting facultative anaerobic bacteria (e.g., *E. coli*) through inter specific competition [83].

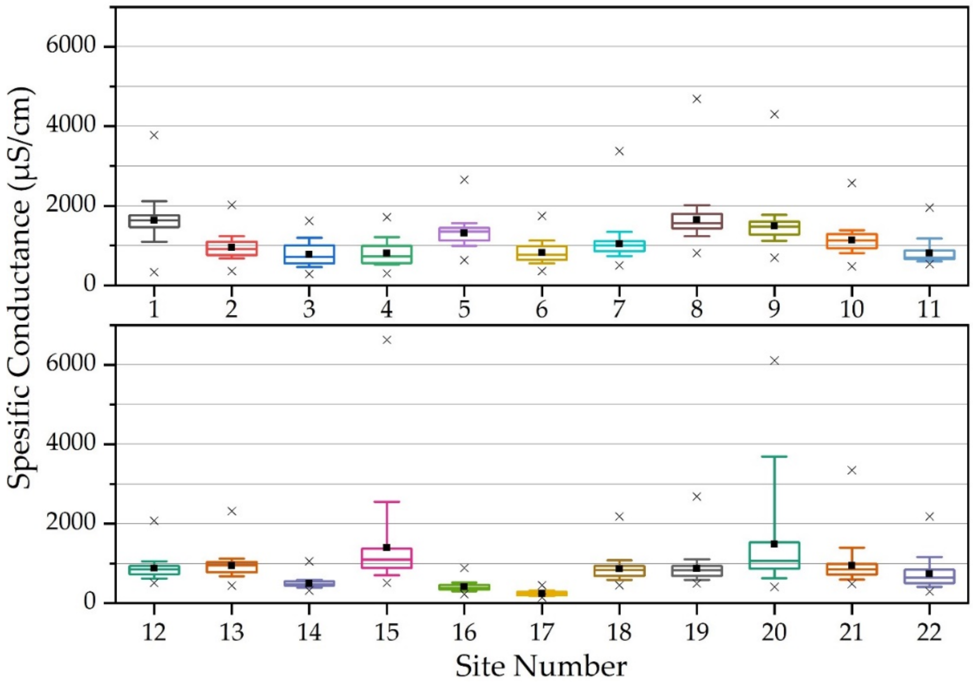

Figure 6. Box and whisker plot of specific conductance (µS/cm) descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

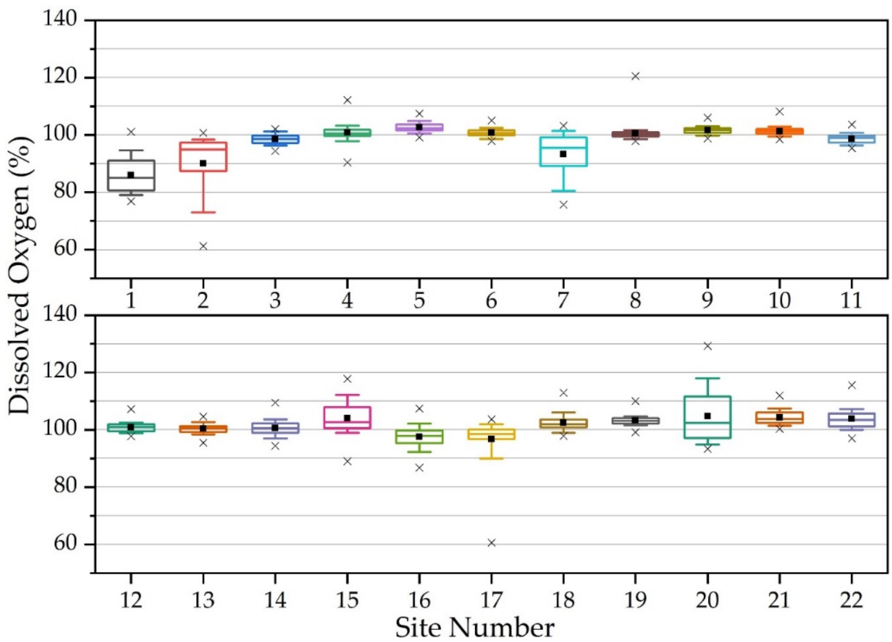

Figure 7. Box and whisker plot of dissolved oxygen (%) descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

Mixed development sub-catchments showed increased chloride ion (Cl^-) concentrations relative to other land use practices. For example, mixed development at site #1 (53%), site #15 (70%), and site #20 (89%) accounted for the highest chloride concentrations among the sampled locations (average concentrations 272.11 mg/L; 220.17 mg/L and 282.87 mg/L, respectively) (Figure 8; Appendix A Table A6). Previous work, using a similar study design, also reported increased chloride relative to increased mixed development land use practices [52]. The application of road salts in mixed development areas has been presented as a contributor to elevated chloride levels in these land use areas [52,84]. Forested land use areas (site #17: 86% forested) comprised the lowest chloride concentrations in WRW, including the lowest average (13.34 mg/L), median (11.79 mg/L), minimum (6.85 mg/L), maximum (39.82 mg/L), and lowest standard deviation (6.13 mg/L) (Figure 8; Appendix A Table A6). Previous work reported similar low(er) chloride concentrations in forested areas relative to other land use types, thereby validating results from the current investigation [52,84,85]. Given the study design and sampling regime, this work shows convincingly that the impact of chloride on microbe concentrations, including fecal microbes, will be increased in mixed development areas.

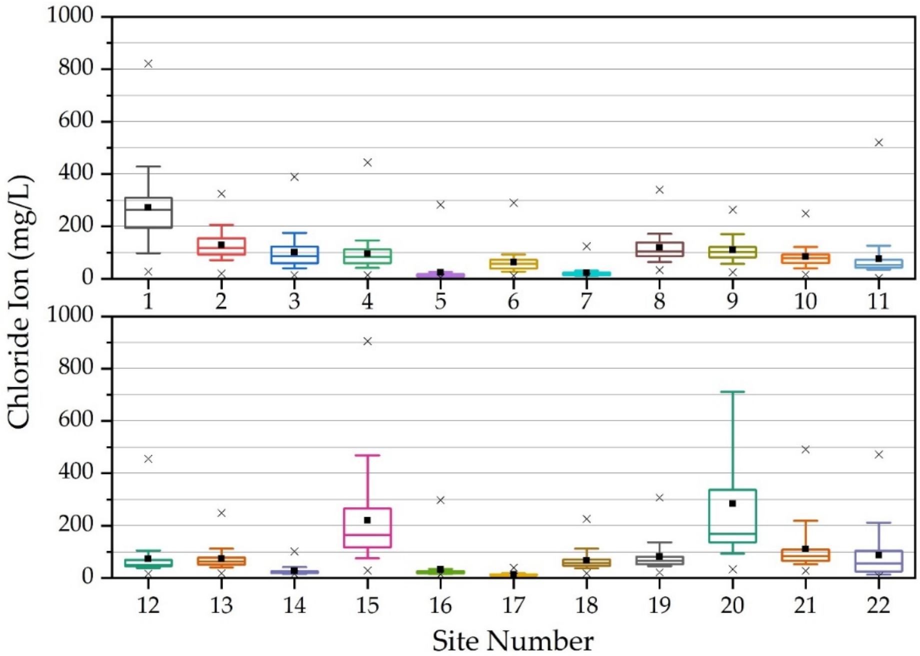

Figure 8. Box and whisker plot of chloride ion (mg/L) descriptive statistics at 22 sampling locations during study period (2 January 2018–1 January 2019) in West Run Watershed, Morgantown, WV, USA. Box delineates 25th and 75th percentiles; line denotes median; square shows mean; whisker describes 10th and 90th percentiles; x shows maximum and minimum when above and below, respectively.

3.4. Annual Non-Parametric Statistical Results

Normality testing showed that annual *E. coli* and physicochemical data were non-normally distributed. Therefore, Spearman's Correlations tests, the non-parametric equivalent of the Pearson's correlations tests [86], were used to investigate annual relationships between *E. coli* concentrations and physicochemical parameters at all 22 sampling locations (Table 2). Results showed that water temperature was significantly ($p < 0.05$) positively correlated with *E. coli* concentrations at 14 (64%) of the 22 sampling locations (Table 2). Six of eight West Run Creek sampling locations (75%) included significant correlations between *E. coli* concentration and water temperature. Despite previous work also reporting spatial and site specific variation regarding water temperature and *E. coli* concentrations [87], water temperature is historically regarded as the primary environmental variable influencing *E. coli* survival in the environment [70]. However, in WRW, pH was significantly negatively correlated ($p < 0.05$) to *E. coli* concentrations at 77% (17 of 22) of sampling locations. Therefore, given the presence of AMD in WRW [6,18,46], which can lower the pH of receiving waters [62] killing (or inactivating) fecal bacteria [63], pH exceeded the influence of water temperatures on *E. coli* concentrations. This is an important finding as it challenges traditional beliefs that temperature is the primary factor influencing the environmental survival of *E. coli*. Moreover, pH values displayed a tipping point (threshold) of between 7.68–7.76, with pH values below this range including significant correlations ($p < 0.05$) with decreased *E. coli* concentrations. Two West Run Creek sites (site #21 and #22) that had insignificant relationships between pH and *E. coli* concentrations were located near the terminus (confluence with the Monongahela River) of the watershed. At these sites, AMD was diluted to levels not influencing *E. coli* survival. Therefore, results also provide evidence for the dilution of AMD impacted waters and subsequent decreased impact on fecal microbe viability. Subsequently, *E. coli* concentrations could potentially be used to assess the freshwater health for aquatic organisms' sensitive to decreased pH and AMD, essentially serving as a bioindicator. Notably, SPC, which is known to be impacted by pH and AMD, displayed significant correlations ($p < 0.05$) with *E. coli* at three sites (#7, #8, and #9) which were particularly heavily impacted by AMD. Generally, SPC did not show consistent correlations with *E. coli* concentrations across the sampling locations, as only 55% (12 of the 22) sites displayed significant relationships. Therefore, based on the Spearman's Correlations tests from the current investigation, SPC was poorly related to *E. coli* CFU's in the current study.

Table 2. Results of Spearman's Correlation test, including annual *E. coli* concentration (colony forming units (CFU) per 100mL) water temperature (°C), pH, specific conductance (SPC; µS/cm), dissolved oxygen (DO; %) and chloride ion (Cl^-; mg/L) at each sampling location (n = 22) during study period (2 January 2018—1 January 2019) in West Run Watershed, WV, USA.

		Site Number										
		#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Water Temp.	SCC	**0.35**	**0.59**	0.20	0.19	**0.75**	**0.37**	0.12	0.26	0.20	**0.50**	**0.52**
	p-value	**0.01**	**0.00**	0.18	0.19	**0.00**	**0.01**	0.43	0.08	0.18	**0.00**	**0.00**
pH	SCC	**0.36**	**0.47**	**0.54**	**0.31**	**0.69**	**0.44**	**0.55**	**0.46**	**0.55**	**0.70**	**0.81**
	p-value	**0.01**	**0.00**	**0.00**	**0.03**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
SPC	SCC	**−0.48**	−0.23	−0.17	−0.18	−0.16	−0.16	**−0.80**	**−0.51**	**−0.55**	−0.14	**−0.41**
	p-value	**0.00**	0.11	0.24	0.21	0.27	0.27	**0.00**	**0.00**	**0.00**	0.33	**0.00**
DO	SCC	**0.31**	−0.14	−0.18	−0.24	−0.01	**−0.39**	−0.16	−0.15	**−0.37**	**−0.40**	**−0.49**
	p-value	**0.03**	0.35	0.21	0.10	0.93	**0.01**	0.27	0.32	**0.01**	**0.00**	**0.00**
Cl^-	SCC	−0.14	−0.20	−0.24	−0.26	−0.16	−0.21	**−0.35**	**−0.45**	−0.28	−0.12	**−0.48**
	p-value	0.34	0.18	0.09	0.07	0.28	0.16	**0.01**	**0.00**	0.05	0.43	**0.00**
		Site Number										
		#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Water Temp.	SCC	**0.71**	**0.66**	**0.71**	**0.37**	0.13	**0.69**	**0.56**	**0.46**	0.05	**0.33**	0.24
	p-value	**0.00**	**0.00**	**0.00**	**0.01**	0.37	**0.00**	**0.00**	**0.00**	0.73	**0.02**	0.10
pH	SCC	**0.62**	**0.74**	**0.69**	**−0.35**	−0.14	0.16	**0.56**	**0.57**	−0.08	0.22	0.25
	p-value	**0.00**	**0.00**	**0.00**	**0.02**	0.36	0.30	**0.00**	**0.00**	0.61	0.14	0.09
SPC	SCC	−0.16	**−0.38**	−0.06	**−0.70**	0.21	**0.30**	**−0.30**	−0.12	**−0.50**	**−0.29**	**−0.33**
	p-value	0.28	**0.01**	0.67	**0.00**	0.16	**0.04**	**0.04**	0.43	**0.00**	**0.05**	**0.03**
DO	SCC	**−0.41**	**−0.56**	0.00	**−0.73**	**−0.47**	**−0.58**	−0.22	−0.20	−0.25	−0.06	**−0.31**
	p-value	**0.00**	**0.00**	0.97	**0.00**	**0.00**	**0.00**	0.14	0.17	0.09	0.68	**0.03**
Cl^-	SCC	**−0.57**	**−0.41**	−0.23	**−0.52**	0.16	−0.15	**−0.44**	−0.27	**−0.37**	**−0.41**	**−0.30**
	p-value	**0.00**	**0.00**	0.13	**0.00**	0.27	0.33	**0.00**	0.07	**0.01**	**0.00**	**0.04**

Note: bold values indicate significant correlations ($p < 0.05$).

Dissolved oxygen (DO) lacked consistent correlations with *E. coli* concentrations, as only 55% (12 out of 22) of sites comprised significant correlations (Table 2). Furthermore, no significant relationship between land use practices and *E. coli* concentrations and DO were found. Previous investigations showed that facultative anaerobic characteristics of *E. coli* decrease its dependence on oxygen for survival [88], which may account for these results (e.g., fish) [89]. Spearman's correlation results between *E. coli* and chloride concentrations showed consistent (with the exception of site #1) significant negative correlations in sub-catchments comprising mixed development land uses in excess of 25% to 30% (Figure 1; Table 2). Analyses also identified that if mixed development land use is less than 25% to 30%, chloride is less likely to influence *E. coli* concentrations in receiving waters. Notably, this tipping point should not be interpreted to imply that lower concentrations are ecologically benign. The insignificant correlation at site #1 may be a function of the relatively small drainage of this sub-catchment (0.30 km^2) and shorter stream distance, relative to other larger catchments with mixed development land use practices in excess of 25–30%. Previous work investigating the influence of mixed development, specifically urban, land use on chloride concentrations reported tipping points approaching 25%; thus, supporting the results from the current investigation [84]. Of importance, the distinct data set of the current work facilitated advanced understanding of the influence of land use practices on *E. coli* concentrations by Cl^- (possible attribute of winter road salting) that may suppress (inactivate or kill) fecal bacteria in receiving waters.

Principal component analysis (PCA) can be implemented to determine which explanatory variables account for the maximal variance in a data set, through the computation of multiple principal components and their respective Eigenvalues [90]. A principal component is defined as a linear function of original data set variables, which maximize variance and is uncorrelated with other principal components [91]. Eigenvalues are used to identify principal components based on the assumption that components comprising the highest Eigenvalues will constitute principal components as Eigenvalues symbolize the variance of the data in that direction [90]. Given most data cannot be accurately described by a single principal component, numerous principal components are typically calculated and ranked based on their Eigenvalues [90]. For the current work, annual PCA results displayed three principal components with Eigenvalues exceeding 1 (an accepted threshold of importance [18,92]). The three principal components comprised Eigenvalues of 2.64, 1.99, and 1.33, respectively, and combined accounted for 66.27% of the variance on the annual data (Table 3). Consequently, the remaining six principal components accounted for only 33.73% of the variance of the data, of which principal component four accounted for 10.91% of the variance. Notably, principal component 4 comprised an Eigenvalue of 0.98, very close to the threshold of importance. For more comprehensive list of the coefficients of the variables comprising the three identified important principal components of the annual PCA please see Appendix A Table A7).

Table 3. Results of principal component analysis comprising 9 variables (*E. coli* concentration, water temperature, pH, SPC, DO, chloride, percentage of agricultural land use, percentage of forested land use, and percentage of developed land use) used to define 9 principal components, displaying eigenvalues, percentage of variance, and cumulative variance during the study period (2 January 2018–1 January 2019) across the 22 monitoring sites in West Run Watershed, West Virginia, USA.

Principal Component	Eigenvalue	Percentage of Variance	Cumulative Variance
1	**2.64**	29.34%	29.34%
2	**1.99**	22.14%	51.48%
3	**1.33**	14.79%	66.27%
4	0.98	10.91%	77.18%
5	0.79	8.80%	85.98%
6	0.61	6.73%	92.71%
7	0.44	4.94%	97.65%
8	0.21	2.35%	100.00%
9	0.00	0.00%	100.00%

Note: bold numbers indicate eigenvalues exceeding 1 (representing importance).

The Annual PCA biplot (Figure 9) compliments the Spearman' correlation results for pH and *E. coli* concentration. Water temperature was also closely correlated to *E. coli* concentrations based on annual PCA results. Both water temperature and pH has been reported to be closely correlated with fecal bacteria concentrations in previous work [9,10,12], thereby supporting results of the current investigation. Historic land use in WRW, specifically mining, influences the pH in the watershed, which is closely correlated with *E. coli* concentrations (Table 2; Figure 9). Moreover, water temperature, which is influenced by land use practices [65–68] is also closely correlated with *E. coli* concentrations. Therefore, annual PCA biplot results emphasize the influence of both historic and contemporary land use practices on fecal bacteria in receiving waters. The biplots of annual land use practices, physicochemical parameters, and *E. coli* concentration relationships facilitates visual assessment of land use impacts on physicochemical parameters, which influences fecal microbes. For example, forested land use showed a negative correlation with water temperature and *E. coli* concentrations (Figure 9) by reduced solar radiation reaching the stream [93]. The decreased water temperatures may suppress *E. coli* concentrations, as the microbe is sensitive to temperature changes [94]. The influence of other physicochemical parameters (e.g., chloride) on *E. coli* concentrations were overshadowed by temperature and pH (Figure 9). Therefore, study results (based on annual average values) highlight temperature and pH as priority factors influencing *E. coli* in the receiving waters of WRW. Notably in watersheds with more neutral pH values and decreased legacy land use impacts (e.g., mining), the influence of physicochemical parameters on *E. coli* concentrations may be different [95]. In the current work, biplot results did not display a strong negative correlation between chloride and *E. coli* (Figure 9). Ultimately, results from the current study highlight legacy land use impacts (mining and AMD) as important considerations regarding microbial water quality management.

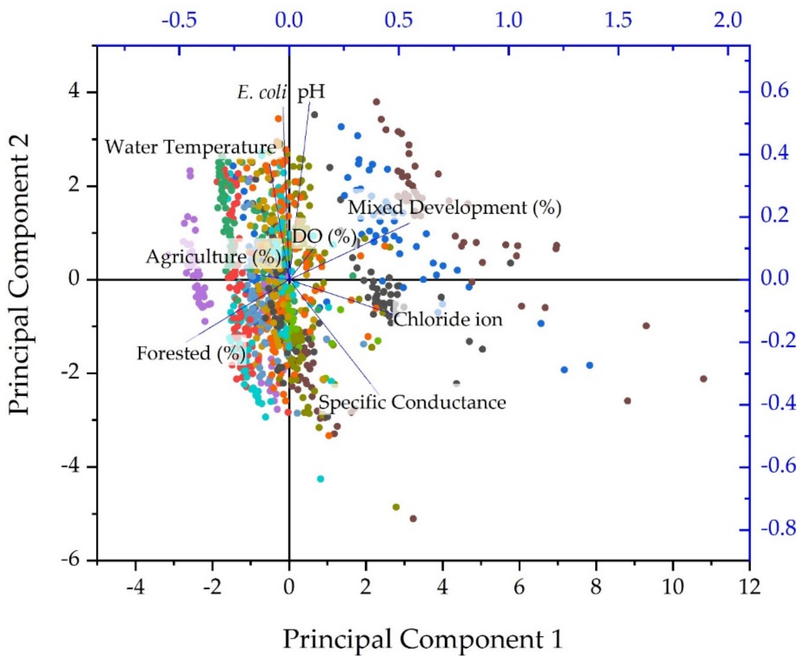

Figure 9. Results of principal components analysis, including biplots, for extracted principal components of annual *E. coli* concentration (CFU per 100 mL), water temperature (°C), pH, specific conductance (µS/cm), dissolved oxygen (DO; %), and chloride ion (mg/L) at 22 monitoring sites (indicated by the different colors) during study period (2 January 2018–1 January 2019) in West Run Watershed, West Virginia, USA.

3.5. Quarterly Non-Parametric Statistical Results

Quarterly PCA results displayed varying Eigenvalues between quarters. Quarter one comprised three principal components, which accounted for 70% of the cumulative data variance (Table 4). Conversely, quarters two, three, and four included four principal components that accounted for 81%, 84%, and 80% for the cumulative variance, respectively (Table 4). The remaining six principal components of quarter one and five, principal components of quarter two to four, did not comprise eigenvalues denoting importance, accounted for only 30%, 19%, 16%, and 20% of the data variance in their respective quarters (Table 4). Appendix A (Tables A8–A11) includes a more thorough presentation of the coefficients comprising the principal components of the PCA for all four quarters.

Table 4. Results of principal component analysis comprising 9 variables (*E. coli* concentration, water temperature, pH, SPC, DO, chloride, percentage of agricultural land use, percentage of forested land use and percentage of developed land use) used to define 9 principal components, displaying eigenvalues, percentage of variance and cumulative variance during quarter one (winter: 2 January 2018–27 March 2018); quarter two (spring: 3 April 2018–26 June 2018); quarter three (summer: 3 July, 2018–25 September, 2018); and quarter four (fall: 2 October 2018–1 January 2019) across the 22 monitoring sites in West Run Watershed, West Virginia, USA.

Principal Component	Eigenvalue	Percentage of Variance	Cumulative Variance	Eigenvalue	Percentage of Variance	Cumulative Variance
		Quarter 1			**Quarter 2**	
1	**3.36**	37.31%	37.31%	**2.81**	31.22%	31.22%
2	**1.53**	16.95%	54.26%	**1.98**	21.97%	53.19%
3	**1.44**	16.00%	70.25%	**1.41**	15.63%	68.82%
4	0.92	10.22%	80.48%	**1.06**	11.74%	80.56%
5	0.73	8.13%	88.60%	0.85	9.39%	89.95%
6	0.53	5.94%	94.54%	0.48	5.36%	95.32%
7	0.26	2.94%	97.48%	0.30	3.38%	98.70%
8	0.23	2.52%	100.00%	0.12	1.30%	100.00%
9	0.00	0.00%	100.00%	0.00	0.00%	100.00%
		Quarter 3			**Quarter 4**	
1	**2.89**	32.07%	32.07%	**2.74**	30.39%	30.39%
2	**2.14**	23.80%	55.88%	**1.87**	20.73%	51.13%
3	**1.34**	14.91%	70.79%	**1.47**	16.30%	67.43%
4	**1.16**	12.92%	83.71%	**1.09**	12.14%	79.57%
5	0.58	6.44%	90.15%	0.79	8.83%	88.40%
6	0.39	4.29%	94.44%	0.57	6.38%	94.78%
7	0.31	3.49%	97.93%	0.29	3.22%	97.99%
8	0.19	2.07%	100.00%	0.18	2.01%	100.00%
9	0.00	0.00%	100.00%	0.00	0.00%	100.00%

Note: bold numbers indicate eigenvalues exceeding 1 (representing importance).

Quarterly PCA biplots (Figure 10) show the predominant influence of pH (presumably AMD driven) on fecal bacteria concentrations in WRW, as pH was closely correlated to *E. coli* concentrations during all four quarters of the study period (2 January 2018–1 January 2019). Conversely, the relationships between *E. coli* concentration and other physicochemical parameters varied between quarters. For example, water temperature was closely correlated with *E. coli* concentrations during quarters two and three, but not during quarter's one and two (Figure 10). The changing relationship between *E. coli* and water temperature is attributable to seasonal changes in air temperature and water temperatures [96]. Colder temperatures are known to suppress *E. coli* concentrations in receiving waters [94]. Thus, temporal and seasonal fluctuations of physicochemical parameters constitute important considerations regarding fecal concentration regimes (and mitigation practices) in receiving waters. The relationships between *E. coli* concentrations and land use practices also lacked consistency (Figure 10). This may have been due to the consistent close correlation between *E. coli* concentrations and pH. Legacy land use practices could be further confounding the influence of contemporary land use practices. Additionally, seasonal variation in land use practices (e.g., application of road salts during winter months) may contribute to the lack of consistent relationships. The high spatial temporal sampling regime and experimental watershed study design in conjunction with quarterly PCA biplots provided new (high-resolution) insight, emphasizing the complexity of temporal changes in *E. coli* concentration, physicochemical parameters, and land use practices. Results can be used by land use managers to inform water quality management strategies during different quarters (seasons), thereby improving efficacy. For example, focusing management strategies, including limiting livestock stream crossings through temporary fencing [97] and irrigation management [98] in quarter one in agricultural areas may be more effective in reducing *E. coli* concentrations than focusing on quarter four in some geographic locations.

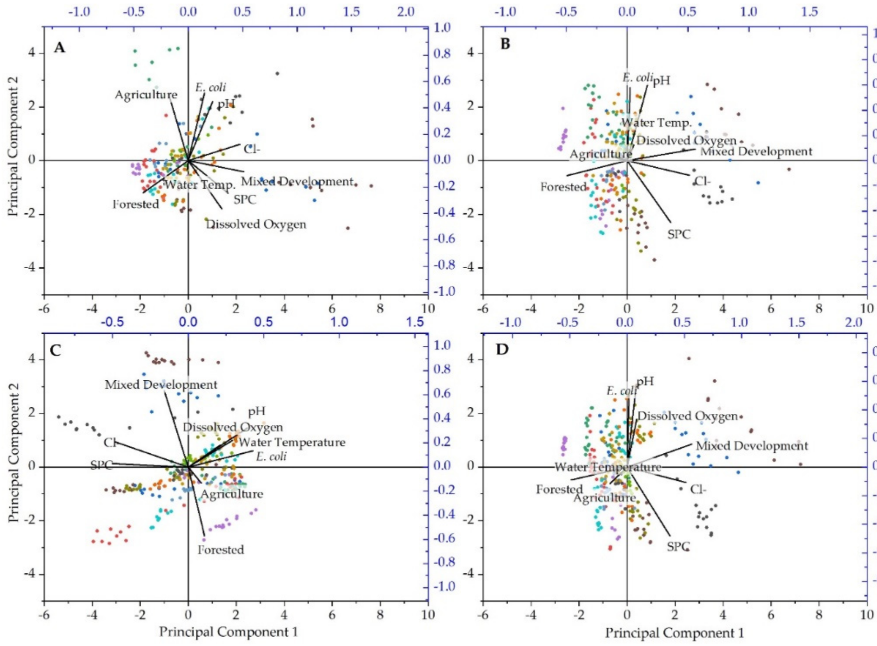

Figure 10. Results of principal components analysis, including biplots, for extracted principal components of quarterly *E. coli* concentration (CFU per 100mL), water temperature (°C), pH, specific conductance (µS/cm), dissolved oxygen (%) and chloride ion (mg/L) at 22 monitoring sites (indicated by the different colors) during study period (2 January 2018–1 January 2019) in West Run Watershed, West Virginia, USA. Note: (**A**) represents quarter one (winter: 2 January 2018–27 March 2018); (**B**) represents quarter two (spring: 3 April 2018–26 June 2018); (**C**) represents quarter three (summer: 3 July 2018–25 September 2018); (**D**) represents quarter four (fall: 2 October 2018–1 January 2019).

3.6. Study Implications and Future Directions

The scale nested experimental watershed study design, calendar-year sampling period, and high spatial and temporal sampling regime used in this work allowed for the identification of legacy land use impacts on *E. coli* concentrations in receiving waters. Legacy land use impacts, specifically mining and subsequent AMD, were identified major influencers of *E. coli* concentrations (and by extension microbial water quality) in receiving waters, even exceeding the influence of water temperature, commonly regarded as the primary factor influencing the environmental survival of *E. coli*. Additionally, the 7.68–7.76 pH tipping point identified regarding the significant correlations between pH and *E. coli* concentrations may indicate the use of *E. coli* as a potential bioindicator species for assessing the freshwater health, specifically in AMD impacted streams. Study results clearly identify legacy land use impacts of mining activity as a major influencer of microbial water quality. A threshold (tipping point) of 25–30% was identified regarding mixed development land use practices and significant ($p < 0.05$) negative correlations between *E. coli* and chloride concentrations. Increased chloride in the receiving waters of mixed development areas has been attributed to road salting [52,84]. This work shows that road salting in mixed development areas exceeding 25–30% total area may impact microbial water quality, through the suppression of *E. coli* concentrations. Future work should include the implementation of similar study design in physiographically dissimilar areas, including simultaneous implementation across different watersheds. This would allow for the comparison of data from climatically distinct regions, further improving understanding regarding physicochemical and land use impacts on *E. coli* regimes. Replication of the experimental watershed study design in areas not effected by AMD could potentially result in the identification of other tipping points regarding land use practices and physicochemical and *E. coli* relationships. For example, the influence of pH in the current work may have obscured the influence of other physicochemical parameters. Future investigations should be undertaken that includes multi-year sampling to better account for annual climate variability.

4. Conclusions

Fecal bacteria concentrations were investigated in a mixed-land-use watershed in the Appalachian region of the eastern United States, using a 22-site nested scale experimental watershed study design. Specific focus was given to the relationships between *E. coli* concentrations, physicochemical parameters (water temperature, pH, SPC, DO, chloride) and land use practices. In the study watershed, there was an approximate 112% increase in *E. coli* concentrations from the AMD impacted headwaters (avg. 177 CFU per 100mL) to the lower portion of the watershed (avg. 376 CFU per 100mL), an approximate 7 Km stream distance. Study results highlight the legacy impacts of historic mining (acid mine drainage) on *E. coli* concentrations, as Spearman correlation test results showed significant correlation ($p < 0.05$) between pH and *E. coli* concentrations at 77% of sample sites. Moreover, a pH tipping point (threshold) in the range of 7.68–7.76 was identified in the current investigation, with pH values below this range including significant correlations ($p < 0.05$) with *E. coli* concentrations. Consequently, pH values in receiving waters below the 7.68–7.76 tipping point will start significantly impacting (decreasing) active *E. coli* concentrations. Furthermore, a land cover tipping point of 25–30% was identified for mixed development land use practices and significant ($p < 0.05$) negative correlations between *E. coli* and chloride concentrations. Therefore, study results indicate that in areas comprising mixed development in excess of 25% to 30%, the application of road salts may suppress fecal bacteria in receiving waters. The importance of seasonal variability on fecal concentrations in receiving waters was illustrated by temporal variability in quarterly PCA biplots of *E. coli* concentrations, physicochemical parameters, and land use practices. The current work advances understanding of land use practice (both historic and current) and physicochemical parameter influences on *E. coli* concentrations in contemporary mixed-land-use watersheds. Results will aid policy makers and land use managers in effective water quality management, in watersheds with fecal contamination challenges.

Author Contributions: For the current work author contributions were as follows: conceptualization, J.A.H.; methodology, J.A.H.; formal analysis, F.P. and J.A.H.; investigation, F.P. and J.A.H.; resources, J.A.H.; data curation, J.A.H.; writing—original draft preparation, F.P. and J.A.H.; writing—review and editing, J.A.H. and F.P.; visualization, F.P. and J.A.H.; supervision, J.A.H.; project administration, J.A.H.; funding acquisition, J.A.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest for the current work.

Appendix A

Table A1. Descriptive statistics of *E. coli* concentration (CFU per 100 mL) at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	170	38	397	429	34	269	84	89	127	210	98
Med.	66	3	260	361	4	194	20	32	25	93	16
Min.	0	0	15	107	0	2	0	0	0	3	0
Max.	1011	961	1011	1011	914	1011	1011	1011	1011	1011	870
Std. Dev.	251	139	315	249	129	276	179	180	241	273	202
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	234	215	457	330	560	206	324	466	415	471	452
Med.	88	91	299	211	575	93	218	436	299	397	397
Min.	0	0	0	5	22	3	0	1	23	2	3
Max.	1011	1011	1011	1011	1011	1011	1011	1011	1011	1011	1011
Std. Dev.	305	266	406	293	373	288	342	339	340	342	345

Table A2. Descriptive statistics of water temperature (°C) at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	11.77	11.09	11.27	11.48	12.28	11.44	12.36	12.32	11.88	11.72	12.84
Med.	11.00	9.90	10.20	9.80	10.85	9.70	11.40	11.30	10.60	10.10	12.80
Min.	5.00	2.60	1.10	−0.10	0.00	−0.10	0.10	−0.10	−0.10	−0.20	2.80
Max.	20.30	19.10	19.50	21.90	23.10	22.10	21.50	21.50	21.80	22.20	22.10
Std. Dev.	4.20	5.51	6.15	6.95	7.73	7.21	6.65	6.28	6.72	7.02	6.22
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	12.10	12.09	12.34	12.86	12.00	11.67	12.53	12.64	12.88	12.87	13.74
Med.	10.70	10.70	10.60	11.40	11.10	10.40	10.90	11.10	11.80	11.60	13.05
Min.	−0.10	0.10	0.30	−0.10	0.10	0.20	−0.10	−0.20	−0.30	−0.20	−0.10
Max.	23.50	22.00	24.00	23.00	24.00	21.70	24.00	24.10	23.50	24.60	27.70
Std. Dev.	7.87	6.91	7.66	6.89	6.99	6.99	7.44	7.72	7.76	7.88	8.61

Table A3. Descriptive statistics of pH at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	7.33	5.94	6.68	7.17	6.19	6.79	5.03	4.37	5.08	5.60	5.56
Med.	7.30	5.62	6.70	7.24	7.17	7.19	5.04	4.23	4.93	5.62	5.70
Min.	6.57	4.74	5.71	6.29	3.05	3.92	2.89	3.05	3.13	3.43	3.08
Max.	8.69	7.93	7.92	7.85	7.74	8.06	7.78	7.58	7.83	7.81	7.66
Std. Dev.	0.44	0.93	0.51	0.42	1.63	0.90	1.43	1.04	1.24	1.08	1.08
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	6.13	6.10	6.54	7.68	7.80	7.86	7.18	7.38	7.97	7.86	7.76
Med.	6.38	6.38	6.75	7.97	7.96	7.96	7.51	7.72	8.11	8.06	7.96
Min.	0.25	3.61	4.01	4.30	4.23	4.00	3.99	4.14	4.27	4.32	4.27
Max.	7.89	7.85	7.76	8.47	8.36	8.45	7.95	8.23	8.65	8.73	8.67
Std. Dev.	1.13	1.07	0.77	0.76	0.58	0.59	0.91	0.92	0.66	0.70	0.72

Table A4. Descriptive statistics of water specific conductance (µS/cm) at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	1639.28	958.51	778.70	810.22	1320.75	827.24	1053.28	1661.13	1497.19	1143.77	812.42
Med.	1643.00	918.00	718.00	727.00	1353.00	769.00	999.00	1562.00	1480.00	1129.00	695.00
Min.	331.60	358.10	286.20	301.80	633.00	354.30	503.00	813.00	688.00	476.60	530.00
Max.	3778.00	2024.00	1620.00	1709.00	2654.00	1745.00	3378.00	4692.00	4302.00	2572.00	1955.00
Std. Dev.	484.90	301.93	305.86	303.74	307.58	263.52	404.69	555.53	496.47	349.67	284.72
	Site Number										
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	885.06	961.58	503.58	1392.64	413.31	249.11	868.99	877.08	1463.58	945.33	738.12
Med.	861.00	958.00	480.40	1116.00	385.00	226.40	845.00	831.00	1045.00	850.00	651.00
Min.	516.00	449.50	321.50	510.00	231.60	144.40	451.60	494.00	408.90	486.50	297.60
Max.	2072.00	2315.00	1052.00	6631.00	890.00	449.90	2184.00	2690.00	6106.00	3345.00	2185.00
Std. Dev.	279.25	292.86	111.90	1041.80	120.58	68.43	306.89	355.26	1193.23	458.84	359.11

Table A5. Descriptive statistics of dissolved oxygen (%) at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	85.93	89.95	98.47	100.78	102.52	100.62	93.20	100.38	101.42	101.16	98.50
Med.	85.10	94.90	98.50	100.50	102.25	100.60	95.00	100.10	101.70	101.30	98.90
Min.	76.90	61.30	93.20	90.40	97.10	95.20	75.70	94.60	94.80	95.30	92.70
Max.	101.10	100.70	102.00	112.10	107.40	105.00	103.20	120.50	106.00	108.10	103.60
Std. Dev.	6.02	10.04	1.97	3.48	1.92	1.68	7.64	3.14	1.60	1.73	1.89
	Site Number										
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	100.76	100.31	100.39	103.78	97.40	96.68	102.30	103.09	104.63	104.22	103.68
Med.	100.90	100.50	100.50	102.70	97.70	98.50	102.00	103.10	102.40	103.80	103.50
Min.	94.00	92.90	93.40	89.10	86.80	60.70	93.80	96.90	93.40	96.70	94.80
Max.	107.20	104.60	109.50	117.80	107.40	103.70	112.90	110.00	129.30	112.00	115.60
Std. Dev.	2.08	2.09	3.05	5.56	3.97	6.60	3.13	1.78	9.06	2.74	3.80

Table A6. Descriptive statistics of chloride ion (mg/L) at each sampling location (n = 22) during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA. Avg. = average, Med = median, Min. = minimum, Max. = maximum and Std. Dev. = standard deviation.

	Site Number										
	#1	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
Avg.	272.11	128.83	101.63	95.87	23.41	62.49	22.53	119.36	109.73	84.04	76.27
Med.	263.40	118.25	86.62	83.73	12.89	57.47	18.25	105.28	102.46	78.75	52.46
Min.	27.08	19.78	13.76	14.31	4.97	12.14	6.38	32.65	24.29	16.35	3.39
Max.	821.26	325.24	388.96	444.44	282.40	289.87	124.07	340.28	263.38	249.22	520.25
Std. Dev.	146.30	55.88	68.56	68.34	45.50	41.00	18.04	55.72	47.16	40.79	84.18
	Site Number										
	#12	**#13**	**#14**	**#15**	**#16**	**#17**	**#18**	**#19**	**#20**	**#21**	**#22**
Avg.	73.42	74.23	27.94	220.17	32.16	13.34	67.52	80.97	282.87	111.21	87.82
Med.	51.01	63.76	22.94	164.83	21.56	11.79	57.96	66.06	168.78	84.08	56.97
Min.	16.48	17.88	15.26	29.23	14.13	6.85	17.69	21.87	33.96	27.24	7.71
Max.	455.64	248.34	101.48	905.31	297.32	39.82	225.82	307.39	1242.68	491.53	472.78
Std. Dev.	74.83	45.41	17.91	176.53	44.33	6.13	40.02	54.18	260.03	95.67	99.41

Table A7. Coefficients of annual principal components comprising 9 variables (*E. coli* concentration, water temperature, DO, SPC, pH, chloride, percentage of mixed developed land use, percentage of agricultural land use and percentage of forested land use) used to define 9 principal components, during study period (2 January 2018–1 January 2019) in West Run Watershed, WV, USA.

Variables	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3
E. coli	−0.03	0.55	0.00
Water Temp	−0.09	0.37	−0.14
DO	0.12	0.11	0.53
SPC	0.41	−0.37	−0.08
pH	0.09	0.57	0.06
Cl-	0.50	−0.12	−0.03
Mixed Development	0.55	0.18	0.08
Agriculture	−0.15	0.02	−0.73
Forested	−0.47	−0.20	0.40

Table A8. Coefficients of annual principal components comprising 9 variables (*E. coli* concentration, water temperature, DO, SPC, pH, chloride, percentage of mixed developed land use, percentage of agricultural land use and percentage of forested land use) used to define 9 principal components, during quarter one (winter: 2 January 2018–27 March 2018) in West Run Watershed, WV, USA.

Variables	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3
E. coli	−0.03	0.55	0.00
Water Temp	−0.09	0.37	−0.14
DO	0.12	0.11	0.53
SPC	0.41	−0.37	−0.08
pH	0.09	0.57	0.06
Cl-	0.50	−0.12	−0.03
Mixed Development	0.55	0.18	0.08
Agriculture	−0.15	0.02	−0.73
Forested	−0.47	−0.20	0.40

Table A9. Coefficients of annual principal components comprising 9 variables (*E. coli* concentration, water temperature, DO, SPC, pH, chloride, percentage of mixed developed land use, percentage of agricultural land use and percentage of forested land use) used to define 9 principal components, during quarter two (spring: 3 April 2018–26 June 2018) in West Run Watershed, WV, USA.

Variables	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4
E. coli	0.03	0.58	−0.09	−0.09
Water Temp	0.05	0.12	−0.12	0.91
DO	0.04	0.09	0.59	−0.18
SPC	0.36	−0.49	−0.08	0.17
pH	0.17	0.60	0.04	0.09
Cl^-	0.51	−0.12	−0.01	−0.11
Mixed Development	0.56	0.09	0.15	−0.01
Agriculture	−0.12	0.03	−0.71	−0.23
Forested	−0.50	−0.12	0.31	0.16

Table A10. Coefficients of annual principal components comprising 9 variables (*E. coli* concentration, water temperature, DO, SPC, pH, chloride, percentage of mixed developed land use, percentage of agricultural land use and percentage of forested land use) used to define 9 principal components during quarter three (summer: 3 July 2018–25 September 2018) in West Run Watershed, WV, USA.

Variables	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4
E. coli	0.43	0.14	−0.29	−0.20
Water Temp	0.21	0.18	0.58	0.33
DO	0.30	0.22	0.48	0.16
SPC	−0.50	0.03	0.24	0.14
pH	0.39	0.32	−0.20	−0.18
Cl^-	−0.48	0.21	−0.13	−0.12
Mixed Development	−0.16	0.63	0.01	−0.15
Agriculture	0.09	−0.13	−0.41	0.78
Forested	0.11	−0.57	0.26	−0.36

Table A11. Coefficients of annual principal components comprising 9 variables (*E. coli* concentration, water temperature, DO, SPC, pH, chloride, percentage of mixed developed land use, percentage of agricultural land use and percentage of forested land use) used to define 9 principal components during quarter four (fall: 2 October 2018–1 January 2019) in West Run Watershed, WV, USA.

Variables	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4
E. coli	−0.03	0.55	0.00	0.21
Water Temp	−0.09	0.37	−0.14	0.79
DO	0.12	0.11	0.53	−0.01
SPC	0.41	−0.37	−0.08	0.19
pH	0.09	0.57	0.06	−0.14
Cl^-	0.50	−0.12	−0.03	−0.17
Mixed Development	0.55	0.18	0.08	0.09
Agriculture	−0.15	0.02	−0.73	−0.44
Forested	−0.47	−0.20	0.40	0.20

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