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Local Bacteriological Survey: Isolation and Characterization of Bacteria in Two  
Lynchburg Parks

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Acceptance of Senior Honors Thesis

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Abstract

Water samples were collected from the bodies of water at Peaks View Park and Percival's Isle Park in Lynchburg, Virginia in two separate trials per collection site. The samples were filtered to isolate bacteria present in the water. The filters were placed on Trypticase Soy Agar (TSA) and Eosin Methylene Blue (EMB) plates and incubated to obtain colony counts of the bacteria. Subsequently, distinct colonies were selected from the filtration plates and streaked for isolation. Isolates were subjected to a variety of analytical methods and thereby identified by genus and species. Fifteen different species were identified from twenty-three different samples. The samples included an alarming number of *E. coli* colonies, as well as other enterobacteria and human pathogens.

Local Bacteriological Survey: Isolation and Characterization of Bacteria in Two

Lynchburg Parks

Summary

Water samples were collected from the bodies of water at Peaks View Park and Percival's Isle Park in Lynchburg, Virginia in two separate trials per body of water. The samples were filtered with sterile metricel filters to isolate bacteria present in the water. The metricel papers were placed alternately on Trypticase Soy Agar (TSA) and Eosin Methylene Blue (EMB) plates and incubated overnight to obtain colony counts of the bacteria. Subsequently, distinct colonies were selected from the filtration plates and streaked for isolation. These bacteria were subjected to a variety of analytical methods and thereby identified by species. Fifteen different species were identified from twenty-three different samples. The samples included an alarming number of *E. coli* colonies, many other enterobacteria and human pathogens, and a large number of colonies of *Azomonas macrocytogenes*.

Introduction

Despite the seemingly idyllic nature of Ivy Creek, at Peaks View Park and the James River basin at Percival's Isle Park, many pathogens lurk in the water. The Virginia Department of Environmental Quality (VDEQ) acknowledges that the waters are "category 5 impaired", due in large part to the use of the river as a sewage overflow site. The VDEQ report cites the presence of *Escherichia coli* and other fecal coliforms as the impairing agents at Ivy Creek and Percival's Isle, and the state planned to take action to purify the waters (Virginia Department of Environmental Quality, 2006). Such

contaminants certainly should pose a concern for any parkgoer who approaches the water, as *E. coli* is the causative agent of severe diarrhea. Fecal coliforms are defined as intestinal-origin Gram-negative, rod-shaped, non-spore forming, facultatively anaerobic bacteria that ferment lactose to produce acid and gas, and are used as an indicator of fecal contamination (Nester et al., 2007).

Though the presence or absence of *E. coli* and other fecal coliforms is of greatest concern for the quality of drinking water, citizens may also be concerned with regard to water in which they may fish, wade, swim, or play. While the VDEQ study provides fecal coliform counts of a wide variety of water sites, its purpose was merely to determine the level of fecal contamination. This study expands upon the work of the Department of Environmental Quality by performing broader analysis to identify all bacteria isolated from James River and Ivy Creek water samples, thereby exposing other microbes unreferenced by the VDEQ study.

## Methods

### *Collection*

For each trial, four 150 milliliter screw-cap glass bottles were sterilized by autoclaving at 121° C, fifteen pounds per square inch for twenty minutes. Two of these bottles were used to collect water at the Percival's Isle/James River site, while the other two were used for collection at the Tenbury Drive entrance to Peaks View Park and Ivy Creek. Samples were collected a maximum of one foot from the shore, at a maximum depth of one foot of water. Two trials were completed for each site, one on October 27, 2007, and the other on January 7, 2008. Both occurred in the early evening.

### *Media Preparation*

Trypticase Soy Agar (TSA) plates were prepared by combining one liter deionized water with forty grams of TSA mix prepared by Becton, Dickinson, and Company (BD-BBL), Lot 6275869. The liquid was autoclaved, poured into sterile plastic Petri plates in fifteen milliliter increments (Kord-Valmark, Lot 8008), and allowed to solidify. TSA (nutrient agar) was chosen based on its properties as a non-selective medium, to demonstrate the presence of bacteria in the environment. It contains beef extract and peptone as nutritional sources (Alexander & Strete, 2001).

Eosin Methylene Blue (EMB) plates were prepared by combining one liter of deionized water with thirty-six grams of EMB mix prepared by Acumedia, Lot 9908-125. The liquid was autoclaved, poured, and allowed to solidify. EMB was selected for its inhibition of gram-positive bacteria by the dyes eosin and methylene blue. Furthermore, EMB demonstrated the degree to which an organism fermented lactose: heavy fermenters (such as *E. coli*) showed a characteristic green sheen, moderate fermenters appeared pink, and non-lactose fermenting bacteria appeared colorless (Alexander & Strete, 2001).

### *Filtration*

Nalgene disposable filter units with .20  $\mu\text{m}$  pores (Lot 626438) under vacuum filtration were used to filter the water. The bacteria were captured on sterile Gelman metricel GN-6 filters, .45 $\mu\text{m}$  (Lot 63077) after passing through a Gelman pre-filter. Fifty milliliters of water sample were passed through each membrane before the membrane was placed onto an agar plate (alternating between EMB and TSA) for culture. Each bottle yielded three membranes, so each trial for each site yielded six membranes (three

TSA, three EMB). The plates were labeled according to trial, bottle number, and collection site and placed in a 37° incubator for twenty-four hours to effect growth.

#### *Colony Count*

After incubation, visibly distinguishable bacterial colonies were identified and the number on each plate was counted. To obtain the number of such bacteria per milliliter of water, the total number counted was divided by fifty milliliters of water that passed through the filter. Colony counts were only obtained from the second trial, as the plates in the first trial were allowed to grow too long and colonies were no longer clearly distinguishable (National Research Council of the National Academics, 2004).

#### *Streak for Isolation*

A representative sample colony of each visually differentiable bacteria was selected using a sterile inoculating loop. Each colony was transferred by streaking an inoculating loop in parallel lines over four quadrants of an EMB plate. The plates were incubated at 37° for twenty-four hours (Leboffe & Pierce, 1996). At this point, initial observations about the shape, color, size and other visual properties of each isolate were recorded.

#### *Gram Stain*

An isolated colony from each streak plate was selected using a sterile inoculating loop and transferred to a clean slide with a drop of sterile water on it. The samples were air-dried onto the slides, and heat-fixed by passing through a Bunsen burner flame several times. Dyes were obtained from BD-BBL. Each smear was covered first with crystal violet dye (Lot 7165771) for one minute and washed with water. Next, the smear was

overlayed with stabilized gram iodine (Lot 7157572) for thirty seconds and again rinsed with water. The smear was next washed with gram decolorizer (Lot 7150659) for ten seconds until no more violet dye was removed. Finally, gram saffranin was placed over the smear for one minute and rinsed with water. Slides were examined under 1000X oil immersion microscopy (Koneman et al., 1988). At this point, the morphology (rods or cocci) and grouping (clusters, chains, pairs, tetrads, etc.) of the cells were recorded as well as the gram stain results. Smears appearing to be predominantly blue or black were labeled as “gram-positive,” and indicated the retention of crystal violet dye in a thick peptidoglycan layer of the cell wall. Predominantly pink or red smears were labeled “gram-negative”; crystal violet was not retained due to a thin layer of peptidoglycan, contained within the outer membrane of the cell wall of gram negative bacteria (Nester et al., 2007). Based on the gram stain and cell morphology results, diagnostic tests were selected on a sample-by-sample basis.

#### *Catalase Test*

Catalase, an enzyme which breaks down hydrogen peroxide, is frequently found in aerobic and facultative anaerobic bacteria because hydrogen peroxide is a potentially toxic byproduct of aerobic respiration. To test for the presence of catalase, isolated colonies were selected and transferred via a sterile inoculating loop to a clean slide. Hydrogen peroxide (3%, ScholAR Chemistry, Lot AD-5209) was applied dropwise to the bacterial colony on the slide. If the bacteria contained catalase, hydrogen peroxide was converted to water and oxygen gas, causing bubbles to appear on the slide. Catalase-negative bacteria produced no reaction (Alexander & Strete, 2001).

### *Oxidase Test*

Cytochrome oxidase is the final enzyme in the electron transport chain of bacterial cell respiration. It oxidizes cytochrome C, the electron transport molecule and reduces oxygen to form water. To test for the presence of oxidase, the reduced reagent N’N’N’N’-tetramethyl-p-phenylenediamine dihydrochloride (Sigma, Lot 51K1684) was suspended to 1% in deionized water and applied to a smear of the colony under investigation on filter paper. If oxidase was present in the colony, it would oxidize the reagent and the colony on the filter paper would turn dark blue within one minute. A negative oxidase test resulted in no color change of the smear (Alexander & Strete, 2001).

### *Aerobicity and Motility Tests*

The motility and aerobicity of bacteria can be determined using a single test medium. Difco manufactures a semi-solid media containing beef extract, peptone, and .5% agar. The lower agar content allows bacteria with flagella for movement to demonstrate their motility by moving away from the stab line in an agar deep (Alexander & Strete, 2001). Motility can be determined by examining the agar deep. Obligate aerobes require an atmosphere containing concentrations of oxygen similar to room air. Microaerophiles require oxygen, but less than that of room air. Anaerobes do not require oxygen for life, and can be either obligate anaerobes, which grow only in anaerobic environments, or facultative anaerobes, which grow in any oxygen environment (Burton & Engelkirk, 2000). In terms of test results, obligate aerobes appear only at the top of the

deep, microaerophiles grow below the top of the stab line, obligate anaerobes grow only in the bottom portion of the stab line, and facultative aerobes grow evenly throughout the stab and on surface of the culture (Cullimore, 2000). Difco bacto-motility test medium (Lot 0105-02) was poured into deeps and allowed to solidify. Deeps were inoculated with a transfer needle and allowed to incubate at 37°C for twenty-four hours before tests were read.

#### *Urease Test*

Certain bacteria possess enzymes called ureases which are capable of hydrolyzing urea to yield alkaline ammonia (NH<sub>4</sub>). The presence of urease is tested for by preparing urea (Sigma, Lot 112F60711) in a broth base containing a pH indicator which is yellow at acidic pH and red at basic pH (McLean & Smith, 1991). Urease liquid media, stored in refrigeration, was poured in three to five milliliter portions into sterile screw-top glass tubes. The tubes were inoculated with the samples in question and allowed to incubate at 37°C for 24 hours, at which point tests were initially read. Pinkish or red tubes were interpreted as positive. Yellow tubes were incubated for another five days at the same temperature. At the end of the five days, if the tubes were pinkish or red they were labeled weakly positive, otherwise they were negative.

#### *Sugar/Substrate Tests*

Many bacteria, particularly within a genus, can only be differentiated on the basis of the sugars they utilized and fermented. To determine if a bacteria utilizes a sugar or substrate, the substance in question is mixed at .5% –1% in phenol red broth (Accumedia, Lot 98C3-122). Bacteria that utilize the sugar or substrate produce acid as a result of

fermentation, causing the tube to turn yellow due to the pH indicator (Singleton, 2004).

Tubes were inoculated with the bacteria in question and incubated for twenty-four hours at 37°C before tests were read. Sugars tested included:  $\beta$ -D-lactose (Eastman, Lot B6F), malonate (Sigma, Lot 102450041), D-maltose (Eastman, Lot A8X), D-mannitol (Fischer, Lot 853323), D-+-mannose (US Biochemical Company, no lot), and meso-inositol (myo-inositol, Sigma, Lot 93F-0516). Other substrates included: alanine (Sigma, Lot 59C-0399), L-ornithine (Difco, Lot 767228), and arginine, (Sigma, Lot 104-F-0611).

#### *Triple Sugar Iron Agar*

Triple Sugar Iron Agar (TSI) is a variation of the broth sugar tests described above. TSI Agar slants contain 2% polypeptone, 1% lactose, 1% sucrose, .1% glucose, phenol red pH indicator, and ferric ammonium citrate. Utilization of sugars proceeds in much the same way as in sugar broth tests, with acid production changing a pH indicator. Specifically, fermentation of lactose/sucrose and glucose causes the entire tube to be yellow. Fermentation of glucose alone causes the butt of the culture to be yellow, but the shallow slant portion turns red as glucose is oxidatively exhausted and peptone is metabolized, producing  $\text{NH}_3$  resulting in an alkaline pH. Gas production during the utilization of sugar is indicated by fissures or pockets in the slant. Some bacteria also produce hydrogen sulfide ( $\text{H}_2\text{S}$ ) by reducing thiosulfate in the medium or breaking down cysteine in the peptone. Ferric ammonium citrate reacts with hydrogen sulfide to produce a black precipitate in the butt of the agar (Leboffe & Pierce, 1995). The slants were made from commercially prepared TSI mix (Difco, Lot 108152JG) and poured to allow the butt of the agar to be four centimeters deep, and the slants were inoculated using an

inoculating needle stab and a fishtail streak of the remaining slant. The slants were incubated for twenty-four hours at 37°C and tests were read for all three sugars as well as gas and H<sub>2</sub>S production.

#### *Methyl Red Test*

The methyl red test is used to determine organisms that ferment glucose to a stable acid end product in a great degree, lowering the pH of the system despite the presence of buffer. Media for the methyl red test (MRVP media) was prepared using mix from Difco (Lot 4131761) and contained peptone, glucose, and a phosphate buffer. Broth was inoculated and incubated at 30°C for five days to allow stable acids to be produced. At the end of the fifth day, methyl red indicator was added. Methyl red indicator is red at pH less than 4.4 and yellow at pH above 6.0, so a red result was labeled positive, and a yellow result negative (Leboffe & Pierce, 1995).

#### *Nitrate Test*

The nitrate test is used to determine whether a bacterial sample has the ability to reduce nitrate to nitrite, free nitrogen gas, or ammonia. Though nitrites are colorless, they react with *N-N*-dimethyl-1-naphthylamine to produce a red color in an acidic environment. Zinc dust reacts with nitrates in an acidic environment to form a similar red color (Gusberti & Syed, 1984). Thus, nitrate test tubes were prepared using premade nitrate broth, inoculated, and incubated at 37°C for twenty-four hours. Following the incubation, several drops of 1-naphthylamine as well as an equal amount of sulfanilic acid were added, mixed, and allowed to stand for 10 minutes. If a red color developed, the bacterial species was known to reduce nitrate to nitrite. If no color developed, zinc dust

was added. If the result was red, no nitrate was reduced; if the result did not change, the organism was nitrate positive, and reduced nitrate to either free nitrogen gas or ammonia.

### *Spore Stain*

Some bacteria are known to form endospores within the cell in response to adverse conditions such as high temperature. The Schaeffer-Fulton procedure is performed to distinguish spore-formers by the use of differential dyes. First, the samples in question were smeared on a slide and heat fixed as described above. The primary stain, malachite green (Difco, Lot 709060) was applied over the smear, which was placed over a boiling water bath to steam and covered with paper towel. The smear was flooded with malachite green for fifteen minutes while heating. At the end of fifteen minutes, the paper towel was removed and the slide was rinsed with water. The slide was then counterstained with saffranin for one minute and again rinsed with water. The slide was dried and microscopically examined; cells with green portions possessed stained endospores, while vegetative cells were pink (Spellman, 2000).

### *Indole Test*

Some bacteria, particularly enteric pathogens such as *E. coli*, are capable of utilizing the enzyme tryptophanase to metabolize the amino acid tryptophan to produce indole. Indole reacts with Kovac's reagent to produce a cherry red color. Kovac's reagent contains HCl and dimethylaminobenzaldehyde (DMABA) dissolved in amyl alcohol, which forms a layer on top of the inoculating medium. This causes the red color to be very easily visible and distinguishable (Leboffe & Pierce, 1995). This experiment was conducted in chambers prepared by BD-BBL in the Enterotube II system (described

below); following incubations of twenty-four hours at 37°C, Kovac's reagent was added to the indole chamber of the Enterotube and read for results.

### *Enterotube II*

The Enterotube consists of a number of different tests compartmentally combined into one convenient tube and is available commercially from BD-BBL. The first chamber tests for the fermentation of glucose, indicated by change from red to yellow, as well as gas production. The second and third chambers test for the presence of lysine and ornithine decarboxylases, respectively, by a change from yellow to purple color. The fourth chamber tests for production of hydrogen sulfide, indicated by a black precipitate, as well as indole production, indicated by a red color when Kovac's reagent is added. The fifth, sixth, seventh, and eighth chambers test for the utilization of adonitol, lactose, arabinose, and sorbitol, all indicated by red to yellow color change. The ninth chamber (not utilized) allows the Vogues-Proskauer test to be performed, to determine production of acetoin. The tenth chamber tests for the fermentation of dulcitol (shift from green to yellow color) and deamination of phenylalanine (black precipitate). The eleventh chamber is a test for urea utilization, and the twelfth chamber tests for growth on citrate media (Alexander & Strete, 2001). The Enterotube (Lot 7025212) was inoculated by touching the needle end of the sterile wire provided with the tube to an isolated colony of the bacteria in question. The needle was pulled through the tube with rotation and pushed back through all chambers. The tube was incubated at 37°C for twenty-four hours, and then test results were read and recorded (Lindh, 1998).

### *Temperature Growth Selection*

Each bacterial species has an optimum, minimum and maximum temperature at which growth occurs. Species can therefore be eliminated and identified based on the temperatures at which they grow or do not grow (Spellman, 2000). The bacteria that underwent temperature selection were inoculated and observed for growth following twenty-four hours at 45°C and/or 4°C, depending on other characteristics known at the time.

### *Blood Agar and Hemolysis*

Some bacteria, particularly *Streptococci* produce hemolysins, compounds which lyse red blood cells in a reaction known as hemolysis.  $\alpha$ -hemolysis results in an incomplete clearing of erythrocytes and cause a greenish color to remain, while  $\beta$ -hemolytic bacteria produce an entire clearing of erythrocytes in the immediate area (Koneman et al., 1998). To test for hemolysis, the bacterial samples in question were inoculated onto commercially-prepared media containing 5% sheep blood (BD-BBL TSA II, lot 7235077) and incubated for twenty-four hours and examined for hemolysis.

## Results

### *Overview*

Listed below are the bacteria identified, a physical description of their appearance, tests which led to their identification, and sites and trials at which they were identified. Colony counts are also given.

*Acinetobacter 10*

This bacterium grew in white colonies approximately one millimeter in diameter on TSA. On EMB, the colonies were a very opaque light pink. The colonies were identified as facultative anaerobic gram-negative coccobacilli. Catalase test was positive. Motility was negative. Glucose, lactose, sucrose, mannitol, mannose, and ornithine utilization tests were all positive. Malonate test was negative. Urease test was negative as was hydrogen sulfide production. Nitrate was reduced to nitrite. Hemolysis was negative. One sample of *A. 10* (as yet an unnamed species) was isolated from trial one of Peaks View Park.

*Azomonas macrocytogenes*

This organism grew in clear to brownish colonies on TSA varying in size from one to three millimeters wide. On EMB, the colonies varied in color from pink to dark purple, occasionally with a light blue opaque line in the center. Colonies on EMB were smooth and shiny. These bacteria were identified as non-motile, facultative anaerobic, gram-negative coccobacilli. Catalase and oxidase tests were positive. Glucose, arginine, ornithine, and malonate tests were negative as was hydrogen sulfide production. Lactose and sucrose utilization varied within strains. Mannitol, trehalose, rhamnose, and maltose tests were positive. Nitrate reduction varied among strains. Urease test was negative. Hemolysis was negative. Three samples of *A. macrocytogenes* were identified from trial two at Percival's Isle, and one sample was identified from Peaks View trial one.

*Azotobacter chroococcum*

This bacterium grew on TSA as shiny, smooth, round, off-white colonies varying from one to three millimeters in width. On EMB, it grew as small pinkish-orange colonies. These gram-negative coccobacilli were determined to be motile facultative anaerobes. Catalase test was positive. Lactose, raffinose, and rhamnose sugar tests were negative. Sucrose, glucose, mannitol, mannose, and maltose tests were positive. Gas production was positive, while hydrogen sulfide production was negative. Urease test was negative. This bacteria was isolated once in Percival's Isle trial one, and once in Peaks View trial two.

*Ensifer adhaerens*

This sample grew on TSA as two to three millimeter wide, rough, raised, dark brown colonies. EMB colonies were fuchsia-colored. These gram negative rods were motile and strictly aerobic. Oxidase and catalase tests were positive. Glucose was not fermented, but was used oxidatively. Xylose, mannitol, maltose tests were positive. Lactose, sucrose, fructose, trehalose, mannose, and adonitol tests were negative. Nitrate test was positive, but not for reduction to nitrite. Urease test was negative. Hydrogen sulfide production was negative. One sample was isolated from Peaks View trial one.

*Enterobacter hormachei*

This sample grew on TSA as shiny brownish/tan colonies with poorly defined edges five millimeters across. On EMB, the colonies were lighter purple with dark purple insets. These gram-negative rods were motile facultative anaerobes. Oxidase and catalase tests were positive. Glucose, rhamnose, xylose, and arabinose sugar tests were

positive. Gas production and hydrogen sulfide production were negative, along with adonitol, lactose, sorbitol, ducitol, and phenylalanine utilization tests. Ornithine carboxylase was positive, while lysine carboxylase was negative. The indole test was negative. The urease test was negative. The citrate test was positive. Nitrate was reduced to nitrite. One sample of *E. hormachei* was isolated from Peaks View trial one.

*Erwinia amylovora*

This bacterium grew on TSA as flat, shiny, beige, circular colonies ranging in size from one to five millimeters. On EMB, colonies grew as pink colonies with a darker purple inner region. These gram-negative rods were determined to be motile facultative anaerobes. Methyl red test was negative. Glucose, was the only sugar tested that was consistently utilized, and did not result in gas or hydrogen sulfide production. Sorbitol and arabinose results varied between strains. Lactose, dulcitol, phenylalanine, mannitol, mannose, and adonitol were all negative. Ornithine decarboxylase was positive, while lysine decarboxylase was positive. The indole test was negative, as was urea. The citrate test was positive. Two samples of *E. amylovora* were isolated, one from Peaks View trial one, one from Percival's Isle trial two.

*Erwinia carotovora*

This sample, grown only on EMB, grew as single colonies approximately one mm in diameter. Colonies were dark purple with a very minor green sheen. These gram negative rods, found in singles and in pairs, were determined to be motile facultative anaerobes. The methyl red test was negative, as was the oxidase test. The catalase test was positive. Glucose utilization, gas production, and hydrogen sulfide production were

positive. Lysine decarboxylase was negative, while ornithine was positive. Lactose, arabinose, and sorbitol sugar tests were positive. Adonitol, dulcitol, and phenylalanine tests were negative. The citrate test was positive, while urease and indole were negative. One sample of *E. carotovora* was isolated from Percival's Isle trial one.

*Escherichia coli*

This bacterium grew on TSA as a white, shiny, smooth colony approximately three millimeters in width. On EMB, it grew as large, smooth colonies surrounded with and covered in a dark, shiny metallic green characteristic of the species. The gram-negative rods in pairs and doublets were determined to be motile facultative anaerobes. The oxidase test was negative, while catalase was positive. The methyl red test was positive. Glucose was positive along with gas production. Hydrogen sulfide production was negative. Lactose was positive, as were arabinose and sorbitol. Adonitol, dulcitol, and phenylalanine were negative. Lysine decarboxylase was positive, while ornithine decarboxylase was negative. Urease was negative, as was citrate. Indole was positive. *E. coli* was the most frequently and widely isolated organism, with one isolate each from Percival's Isle trials one and two, and two from Peaks View trial two.

*Klebsiella oxytoca*

This bacterium grew on TSA as smooth, shiny, round off-white colonies one millimeter or less in diameter. On EMB, the colonies were burgundy colored. These non-motile, gram-negative, rod-shaped facultative anaerobes were oxidase negative. The methyl red test was also negative. The glucose test was positive and produced gas. Adonitol, lactose, arabinose, sorbitol, and dulcitol tests were all positive as well. Lysine

decarboxylase was positive, while ornithine was negative. The hydrogen sulfide test was negative. Citrate and indole were both positive while phenylalanine was negative and urease was weakly positive. One *K. oxytoca* sample was isolated from Peaks View trial one.

*Kluvenya ascorbata*

On TSA this organism grew as dull, rough, white colonies of one millimeter in diameter. EMB colonies were shiny and light purple. These gram-negative rods were determined to be motile facultative anaerobes. The oxidase test was positive. Glucose was positive and produced gas. Lysine and ornithine decarboxylases were both positive. Hydrogen sulfide production was negative. Indole was positive. Adonitol, lactose, dulcitol, and phenylalanine were all negative. Arabinose and sorbitol sugar tests were positive. Urease test was negative and citrate test was positive. One sample of *K. ascorbata* was identified in Peaks View trial one.

*Micrococcus sedentarius*

This organism grew on TSA as off-white, shiny, flat, and smooth colonies with diameters of approximately one millimeter each. On EMB, colonies were dark purple. These tiny gram-positive cocci were determined to be facultative anaerobes. The oxidase test was negative. The catalase test was positive. The glucose test for fermentation was negative, as was nitrate reduction. One *M. sedentarius* isolate was identified from Percival's Isle trial one.

*Pseudomonas diminuta*

On TSA, *P. diminuta* grew as whitish, smooth, raised, shiny colonies 3–4 mm in diameter. EMB colonies varied in color from pink to dark blue. These gram-negative coccobacilli were non-motile facultative anaerobes. Catalase and oxidase tests were both positive. Lactose, glucose, sucrose, mannitol, malonate, trehalose, and arginine sugar tests were all negative. Hydrogen sulfide production was negative, as was nitrate reduction. Maltose and raffinose sugar tests were positive. The urease test was negative. Growth did not occur at 4°C but did occur at 41°C. One *P. diminuta* sample was identified from Peaks View trial one.

*Pseudomonas pseudomallei*

This organism grew on TSA as a pale/whitish color in smooth, shiny colonies three to five millimeters wide. On EMB colonies had an orange/pink outer opaque portion and an inner fuchsia portion. The sample was identified as gram-negative coccobacilli. Motility test was negative, and the sample was determined to be composed of facultative anaerobes. The catalase test was positive, as was the oxidase test. Lactose/sucrose, glucose, trehalose, ribose, mannitol, and adonitol tests were all positive. Malonate and xylose tests were negative. Nitrate was reduced to nitrite. The urease test was negative, as was hydrogen sulfide production. One *P. pseudomallei* isolate was identified from Peaks View trial two.

*Pseudomonas stutzeri*

This organism grew as shiny colorless colonies less than one millimeter wide on TSA. On EMB, the colonies were dark purple. These gram-negative coccobacilli were

present in doublets. Tests showed the sample to be composed of non-motile facultative anaerobes. Catalase and oxidase tests were positive. The urease test was negative, as was hydrogen sulfide production. Nitrate was reduced to nitrite. The bacteria utilized glucose and mannitol. Lactose, sucrose, trehalose, adonitol, xylose, malonate, meso-inositol, and alanine were all unutilized. The organism grew at 41°C. One sample was identified as *P. stutzeri* from the second Percival's Isle trial.

*Staphylococcus capitis, subspecies ureolyticus.*

This organism grew as a smooth, colorless colony varying in width from two to four millimeters on TSA. On EMB, the color varied from maroon to fuchsia and a very slight green sheen was visible in the background but not on the bacteria proper. The gram-positive cocci were motile facultative anaerobes. The catalase test was positive, while the oxidase test was negative. Maltose, lactose, mannose, galactose, ribose, and sucrose were all positive. Xylose, arabinose, and trehalose were negative. The urease test was positive. Nitrate reduction occurred. One *S. capitis* isolate was obtained from Peaks View trial two.

*Peaks View Colony Counts*

Three plates of TSA were counted for this location. Unfortunately, due to time constraints the colonies were classified by color rather than by species identification. For the first plate, six yellow colonies were counted, six orange, thirteen white, fifty pale pink, and fifty beige. On the next plate five yellow colonies were counted, thirty orange, six white, 300+ pale pink, and 300+ beige. On the last plate, one yellow colony was counted, thirty orange, twelve white, 170 pale pink, and 167 beige. This resulted in TSA

averages of four yellow colonies, twenty-two orange colonies, ten white colonies, 173 pale pink colonies, and 172 beige colonies per fifty milliliters of water. In total, there was an average of 389 colonies/50 mL or 778 colonies/100 mL of Peaks View Park water filtrated (see Table 1).

**Table 1. Colony counts on TSA per 50 mL of water filtered for Peaks View Park.**

Plate Number	Yellow	Orange	White	Pale Pink	Beige	Total
1	6	6	13	50	50	125
3	5	30	6	300+	300+	640+
5	1	30	12	170	167	380
Average	4	22	10.333	173.333	172.333	382

Three plates of EMB were also counted and presumably identified by species. However, some species died before identification was complete and some species that appeared differently on the counting plate resulted in the same species being identified from different isolates. Such convergent species were labeled according to the plate onto which they were streaked (“A” and “F,” respectively). On the first plate, 106 *E. coli* “A,” 114 *S. capitis*, twenty *P. pseudomallei*, 242 PVP2D (unidentified), twenty-five *A. chroococcum*, and zero *E. coli* “F” were counted. On the next plate, forty-five *E. coli* “A,” fifty-six *S. capitis*, seven *P. pseudomallei*, 200+ PVP2D, and one *E. coli* “F” colony were isolated. Forty-five *E. coli* “A,” forty-five *S. capitis*, nine *P. pseudomallei*, 277 PVP2D, thirty-five *A. chroococcum*, and one *E. coli* “F” were counted from the final plate. This resulted in an average of sixty-five *E. coli* “A” colonies, seventy-two *S. capitis* colonies, twelve *P. pseudomallei* colonies, 240 PVP2D colonies, thirty *A. chroococcum* colonies, and one *E. coli* “F” colony per fifty milliliters of water. The

combined *E. coli* average was 66 colonies/50 mL or 132 colonies/100 mL. An average total of 419 colonies/50 mL of water or 838 colonies /100 mL of water was obtained (see Table 2).

**Table 2. Colony counts on EMB per 50 mL of water filtered for Peaks View Park.**

Plate Number	<i>E. coli</i> * (A)	<i>S. dephini</i>	<i>P. pseudo-mallei</i>	PVP2D (un-identified)	<i>A. chroococcum</i>	<i>E. coli</i> * (F)	<i>E. coli</i> * (A+F)	Total	*Total coli-forms
2	106	114	20	242	25	0	106	507	106
4	45	56	7	200+	30	1	46	339+	46
6	45	45	9	277	35	1	46	412	46
Average	65.333	71.667	12	239.667	30	.667	66	419.333	66

*Percival’s Isle Colony Counts*

Again three TSA colony count plates were obtained, identified by appearance. On the first plate two yellow colonies, forty-two white shiny colonies, eleven white dull colonies, 148 pale pink colonies, seven orange colonies, and zero green colonies were counted. On the next plate five yellow colonies, 140 white shiny colonies, thirty-six white dull colonies, 152 pale pink colonies, eleven orange colonies, and two green colonies were counted. On the last plate twenty yellow colonies, seventy-two white shiny colonies, twenty-six white dull colonies, sixty-three pale pink colonies, seven orange colonies, and zero green colonies were isolated. This resulted in an average of nine yellow colonies, eighty-five white shiny colonies, twenty-four white dull colonies, 121 pale pink colonies, eight orange colonies, and one green colony per fifty milliliters of

water. In total, an average of 248 colonies/50 mL of water or 496 colonies/100 mL of water grew on TSA (see Table 3).

**Table 3. Colony counts on TSA per 50 mL of water filtered for Percival's Isle.**

Plate Number	Yellow	White, Shiny	White, Dull	Pale Pink	Orange	Green	Total
1	2	42	11	148	7	0	210
3	5	140	36	152	11	2	346
5	20	72	26	63	7	0	188
Average	9	84.667	24.333	121	8.333	.667	248

Three EMB plates were also prepared for colony counts. Again, several colonies appeared physically different and were identified as the same species; they are denoted here by a letter to distinguish attempted isolation (“B,” “C,” and “E”). For the first plate, fifty colonies of PI2A (unidentified), 126 colonies of *A. macrocytogenes* “B,” forty-three colonies of *A. macrocytogenes* “C,” fifty-two colonies of *P. stueri*, sixty-three colonies of *A. macrocytogenes* “E,” and zero colonies of *E. coli* were counted. Twenty-eight colonies of PI2A, six colonies of *A. macrocytogenes* “B,” three colonies of *A. macrocytogenes* “C,” eight colonies of *P. stutzeri*, one colony of *A. macrocytogenes* “E,” and zero colonies of *E. coli* were counted on the next plate. On the final plate, five colonies of PI2A, zero colonies of *A. macrocytogenes* “B,” one colony of *A. macrocytogenes* “C,” four colonies of *P. stutzeri*, two colonies of *A. macrocytogenes* “E,” and three colonies of *E. coli* were counted. This resulted in an average of twenty-seven colonies of PI2A, forty-four colonies of *A. macrocytogenes* “B,” sixteen colonies of *A. macrocytogenes* “C,” twenty-one colonies of *P. stutzeri*, twenty-two colonies of *A. macrocytogenes* “E,” and one colony of *E. coli* for every fifty milliliters of water

filtrated. The combined total of *A. macrocytogenes* was forty-nine colonies for every fifty milliliters of water. The average total number of colonies grown on EMB was 132/50 mL or 263 colonies/100 mL of water (see Table 4).

**Table 4. Colony counts on EMB per 50 mL of water filtered for Percival's Isle.**

Plate Number	PI2A (un-identified)	<i>A. macrocytogenes</i> "B"	<i>A. macrocytogenes</i> "C"	<i>P. stutzeri</i>	<i>A. macrocytogenes</i> "E"	<i>E. coli</i> *	<i>A. macrocytogenes</i> ("B" + "C" + "E")	Total	*Total Coli-forms
2	50	126	43	52	63	0	106	334	0
4	28	6	3	8	1	0	37	46	0
6	5	0	1	4	2	3	3	15	3
Average	27.667	44	15.667	21.333	22	1	48.667	131.667	1

## Discussion

### Overview

The bacteria identified are described giving particular attention pathogenicity and environmental problems. Overall trends are also analyzed with respect to the various trials for the separate parks as well.

### Peaks View Park

There was marked diversity among the bacteria isolated from Peaks View Park, with no overlapping species between trials one and two. This may be due to variation in amount of soil contained in the water collection. It may also partially be due to the researcher's skill in isolation, as the growth on colony count plates was difficult to distinguish when streaking for isolation. Furthermore, the diversity could be due to the weather and season during which the samples were taken: trial one occurred at the

beginning of the fall, during a period when temperatures reached 70 degrees Fahrenheit, while trial two occurred mid-winter, in sub-forty degree weather. Also, the diversity of bacteria between trials may be due to the intermittent nature of the sewage overflow into the James River. Peaks View Park's Ivy Creek would be affected by a sewage overflow event after the event initially occurred at its contributor; if sewage initially was dumped into the James River proper, it could take several weeks for the bacterial effects to be observed at the downstream offshoot in Peaks View Park. Isolates from Peaks View Park are described below.

*Acinetobacter 10.*

This species of bacteria, yet to be named, is naturally found in soil, water, and sewage (Holt et al., 1994). Acinetobacters are also receiving increased attention due to their recovery in hospital settings. These organisms may function as opportunistic pathogens, infecting the respiratory tract, urinary tract, and most importantly for this study, wounds. Treatment may become problematic because many of these organisms are antibiotic resistant (Murray et al., 2002).

*Azomonas macrocytogenes.*

This species of bacteria, also naturally occurring in soil and water, is non-pathogenic. Known for its ability to fix nitrogen, *A. macrocytogenes* has also recently been recognized as capable of degrading polyacrylamide, a component of plastic (Nakamiya & Kinoshita, 1995).

*Azotobacter chroococcum.*

This soil and water dwelling organism is also non-pathogenic. *A. chroococcum* is best known for its use in soil remediation, as it is capable of fixing large quantities of nitrogen (Wise, 2000).

*Ensifer adhaerens.*

This soil isolate is non-pathogenic to humans as well. It is known to act as a predator against other soil bacteria (Germida & Casida, 1983).

*Enterobacter hormachei.*

This bacteria is a fecal coliform, normally residing in the intestinal tract but widespread throughout the environment due to human excretion. Studies have shown that *Enterobacter* species are common causes of nosocomial urinary tract, wound, and bloodstream infections, as well as occasionally causing hospital-acquired pneumonia (Murray et al., 1999).

*Erwinia amylovora.*

Found in water and sometimes carried by insects, *E. amylovora* poses no threat to humans. However, the bacterium is responsible for a plant disease known as “fireblight,” which causes the wilting of branches and sometimes the death of trees in as little as six weeks. Pears and apples are known to sometimes suffer the effects of *E. amylovora* infection (Adams & Early, 2004).

*Escherichia coli.*

This bacterium is used as the indicator for fecal contamination in water, and non-pathogenic strains of the organism predominate. However, certain strains of *E. coli* can

also cause enteric and extraintestinal infections. Enterotoxigenic *E. coli* (ETEC) is the cause of watery diarrhea in children and travelers in developing countries. ETEC act by penetrating the gut's mucous layer and adhering to epithelial cells to interfere with sodium pump mechanisms. This leads to the accumulation of water and sodium ions in the lumen of the gut. At least two toxins (heat-labile toxin and heat-stable toxin, LT and ST) are produced. Verotoxin-producing *E. coli* (VTEC) is the form of *E. coli* frequently described in newscasts, causing rapid-onset watery diarrhea by serotype *E. coli* O157:H7. Verotoxins work by adhering to the gut and killing the cell by interfering with ribosome function. Enteroinvasive *E. coli* (EIEC) cause diarrhea accompanied by abdominal pain and fever. This form of *E. coli* infection may be diagnosed by blood, mucus, and leucocytes in the stool. Enteropathogenic *E. coli* (EPEC) causes diarrhea and vomiting and is transmitted by contaminated food and water. The pathogenic effects of this infection are caused by direct interference with mucosal cell function, rather than by toxin production (McLean & Smith, 1991).

*Klebsiella oxytoca.*

*K. oxytoca* is a fecal coliform known to be a pathogen in many long-term-care and hospital settings. Normally spread person-to-person, infection with this organism can cause extraintestinal infections similar to those caused by *E. coli*. Some antibiotic-resistant strains exist, and the most common clinical syndromes are pneumonia, abdominal infection, UTI, wound infection, soft tissue infection, and bacteremia (Kasper & Harrison, 2005).

*Kluyvera ascorbata.*

This bacterium naturally occurs in soil and certain strains have been characterized as significantly enhancing plant growth in heavy-metal contaminated soils (Ma, Zalec, & Glick, 2001). *K. ascorbata* has also been known to cause infections among humans in a variety of sites, excluding the brain. *K. ascorbata* opportunistic infection is rare, but very serious. *Kluyvera* are ampicillin resistant (West, Vijayan, & Shekar, 1999).

*Pseudomonas diminuta.*

This organism is widespread in the natural environment and poses little known threat to humans. Many proteins of this species have been isolated and characterized, and it is utilized as a test of the accuracy of sterilization by filtration, as it can pass through a .45 µm pore (Russell et al., 2004).

*Pseudomonas pseudomallei.*

This bacterium, also widespread in nature, is not pathogenic to humans. However, it is occasionally known to cause Whitmore's disease (melioidosis) in rodents (Turkington & Ashby, 1998).

*Staphylococcus capitis.*

Usually associated with the skin and mucous membranes of vertebrates, *S. capitis* can also be isolated from food products and water. Infection from this coagulase-negative staph is rare, but has occurred, most notably in patients with pacemaker electrodes (Honeyman, Friedman, & Bendinelli, 2002).

*Percival's Isle*

The Percival's Isle trials showed marked diversity similar to the trials of Peaks View, though in this case *E. coli* was isolated in each trial. The diversity of bacteria between trials could be due a variation in the percentage of soil content in each trial, or to the weather variations and seasons of the year during which samples were taken, as described above.

*Azomonas macrocytogenes.*

For description see Peaks View Park section above.

*Azotobacter chroococcum.*

For description see Peaks View Park section above.

*Erwinia carotovora.*

*E. carotovora* is normally isolated from both water and soil. Harmless for humans, this plant pathogen is responsible for soft rot of potatoes, carrots, bulbs, and irises while in storage (Adams & Early, 2004). Interestingly, this bacteria has also shown to be a source of asparaginase, which is used to treat acute lymphoblastic leukemia with minor side-effects (Pandey, 2004).

*Escherichia coli.*

For description see Peaks View Park section above.

*Micrococcus sedentarius.*

This bacterium can be found in soils and fresh water as well as on the skin of humans and other animals. *M. sedentarius* is an indicator of spoilage in many meats (Blackburn, 2006).

*Pseudomonas stutzeri.*

*P. stutzeri* is ubiquitous, found in soil, water, and hospital environments. Though it is generally thought to be harmless, this bacterium can cause severe community acquired pneumonia in immunocompromised patients. Some joint, skin, and eye infections have also been described (Loyse, Storrington, & Melzer, 2006).

*Conclusion*

From the types and numbers of pathogens and enteric bacteria isolated in these trials it should be clear that to maintain health, one should avoid contact with contaminated water at both Peaks View Park and Percival's Isle. Oddly enough, despite its distance from the actual sewage dumping site, the Peaks View Park collection location contained far more bacteria than the Percival's Isle location did. Applying VDEQ standards of less than 200 fecal coliforms and 100 *E. coli* per 100 milliliters of water, only exposure to the waters examined in Peaks View Park the study can officially be considered a health hazard (VDEQ, 2007). However, the limited nature of the number of trials of colony counts would probably implicate the Percival's Isle site as well, due to the diversity and multiplicity of samples found in the first trial.

In future studies, a greater focus on colony counts would be productive. While the current study measured colony counts for some of the bacteria, its conclusions were limited by the number of trials performed. In a future systematic study, weather and seasonal patterns can be either controlled for or analyzed at length. Serotyping of the isolates could also prove productive, to determine if *E. coli* and other coliforms isolated were pathogenic or merely indicators of fecal matter.

The variety of non-fecal pathogens isolated from the water sources suggests that VDEQ would do well to expand its criteria for compromised water beyond analysis of fecal coliforms to include such infectious agents as *P. stutzeri* and *Acinetobacter 10*. However, the expansion of impaired water standards should remain a lower priority than the initial cleanup of the water, as fecal coliforms pose a greater threat to the health of exposed individuals than the other isolates.

Although full-blown outbreaks of waterborne disease associated with recreation are infrequent, many occur in isolation (Cabelli, 1978). Furthermore, human fecal pathogens may lodge in free-swimming fish, posing a threat to the human population that eats the fish (Mitchell, 1978). In addition to avoiding the contact with the contaminated waters themselves, proactive parkgoers may wish to lobby the appropriate authorities for proper treatment of the sewage rather than releasing raw overflow into public waters. Studies have shown that storing raw sewage in retention ponds for a month may reduce total coliforms by up to 99.9% (Geldreich, 1972), resulting in safe water and a healthier day at the park.

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