Effects of Human Presence and Fecal Contamination on Bacterial Distribution in Streams of Glacier National Park

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ABSTRACT: Abundance of four key bacterial groups and *Escherichia coli* were compared along the stream continuum, above and below point source contamination, in high-impact and low-impact streams in Glacier National Park. Point source fecal contamination was found to greatly increase the concentration of *E. coli* for approximately 1500 m past the point source, where it dissipated back to normal levels. The pollution exhibited a negative correlation to the distributuion of the genera *Xanthomonas*, but little correlation in other groups. High human activity increased coliform abundance, but did not necessarily affect the other species in the stream.

KEY WORDS: Bacterial distribution • Coliforms • Pollution • Water quality • Anthropogenic impact

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INTRODUCTION

The bacterial biodiversity of a stream is vital to its ecological stability and functionality (van der Zaan et al. 2010). Bacteria play an essential role in nutrient cycling, serving as the main source of nitrogen and carbon for higher order organisms, the removal of halogens and metals from pristine waters, detoxification of urea, primary production, and provide the base of stream food webs (Dang et al. 2010, Findley 2010, van der Zaan et al. 2010). The relative abundance and functional types of species in a stream have been shown to be affected by pH, organic content, temperature, dissolved oxygen content, and mineral composition (Dang et al. 2010, Battin et al. 2000, Feris et al. 2003, McFeters et al. 1978). The presence of humans around pristine streams has been shown to have a significant effect on streams' natural biota (Olapade et al. 2005, Suart et al. 1976).

In the absence of algal blooms, heterotrophs are dependent upon allochthonous carbon that enters sporadically and at lower rates. As algal blooms increase downstream due to fewer velocity disturbances and higher nutrient input from surrounding fauna or anthropogenic inputs, the amount of available carbon to support bacterial growth also increases (Battin et al. 2000). This signifies that the specific growth rate and, therefore, the bacterial carbon production increase with greater distances from the headwaters in a natural system. In high altitude areas that have less of a allochthonous carbon input, a greater network of carbon and nitrogen fixing bacteria are necessary to compensate for significant fluctuations in nutrient availability and the colder temperatures that disturb membrane transport protein efficiency (Rompré et al. 2002).

3 Bacterial distribution and fecal contamination

While many studies have explored the presence of fecal bacteria as an indicator of stream health, few have looked at the effects of those bacteria on the biodiversity of naturally-occurring stream bacteria (Lamka et al. 1990, Lear et al. 2009, McFeters et al. 1978, Olapade et al. 2005, Rompré et al. 2002, Surbeck et al. 2009). Pollution from waste water treatment plants has been shown to add large amounts of organic matter and nutrients to the water in ratios that can kill off sensitive bacteria and promote the growth of tough bacteria, kicking many cycles into overdrive and leading to the deposition of heavy metals from their use in place of carbon in the metabolism of the bacteria (Dang et al. 2010, Findley 2010, van der Zaan et al. 2010). The introduction of human waste has given rise to bacteria that are increasingly resistant to antibiotics starting from the pollution source and downstream (Harwood et al. 2000, McArthur and Tuckfield, 2000).

The presence of coliforms could have a drastic effect on keystone species because coliforms and most nutrient-cycling bacteria are facultative anaerobes, utilizing the same habitat and resources (Schneider and Topalova 2009). A large portion of the vital bacteria for the cycling of organic matter form biofilms on the rocks in a stream (Lear et al. 2009, Olapade et al. 2005, Schneider and Topalova 2009), but it has been found that coliforms can successfully outcompete biofilm-forming bacteria and form their own slime layers using the natural biofilm as a backbone to establish its matrix (Rompré et al. 2002). The choking-out effect that pollution has on biofilms disrupts their ability to function as a transient storage unit and production unit of carbon, and disrupts their ability to properly remove nitrate and ammonia along the stream continuum (Battin et al. 2000).

In Glacier National Park, managers have removed many areas of direct fecal contamination, but pit toilets still exist alongside streams in established backcountry campgrounds and high-traffic trails. These toilets are drained by the park, but there is no barrier in the soil between the fecal matter and the river bed. In frontcountry campgrounds, septic systems hold the waste in tanks, but these could be leaking after several winters of frost heave. While simple tests have found fecal indicator bacteria, or coliforms, are present in the streams, there is no quantification of the amount of contamination or its effect on the microbial biodiversity of these streams. The bacterial population has been shown to differ for each stream, but a base of bacterial groups common to the streams in Western Montana include the Pseudomonades and the genera *Aquabacterium*, *Rhodoferax*, *Hyphomicrobium*, *Nostoc, Chamaesiphon* and *Pirellula* (Feris et al. 2003).

The purpose of this study was to quantify the variability in these five basal groups in comparison to levels of coliforms before and after known point sources of contamination. The bacterial levels were determined using real-time qPCR and interpreted through analysis of varience (ANOVA) above and below the point source. The data suggests that bacterial population are either affected by very small changes in *Escherichia coli* levels or other forcings caused from human contact and pollution.

MATERIALS AND METHODS

Study Streams

Three streams were chosen to represent the different stream conditions present in Glacier National Park. The first represented a high-impact stream which

has heavy human traffic, a pit toilet near the mouth of the stream and a campground at the bottom. The second was a high-impact stream that has high contact in two areas, a campground near the bottom and a large falls further up, with no contact in between. The final stream was a control backcountry stream, with no human contact at any point. These streams had similar riparian growth overhanging the edge of the stream and similar drop-pool topographies. Samples were taken approximately one-third of the distance across the stream, as it was found in a pilot study that the bacterial communities did not differ significantly from the edge of the stream to the middle of the path flow. Sample collection started at the stream confluence and continued to the approximate headwaters or areas above considerable human impact for each stream.

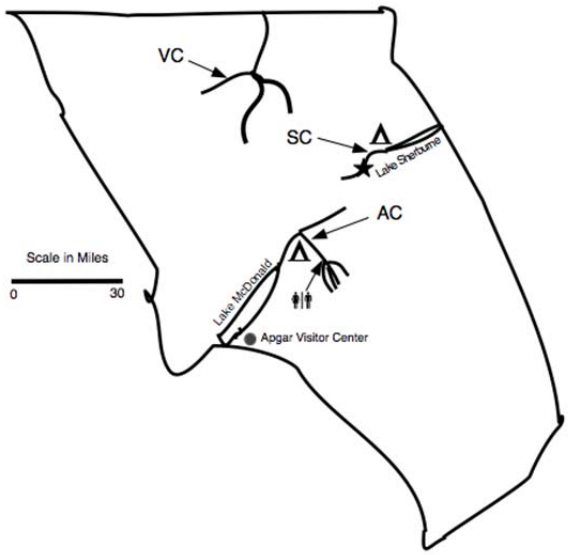


Fig. 1. Study creeks in Glacier National Park and approximate location of point source contamination. Avalanche Creek, AC, Swift Current Creek, SC, and Valentine Creek, VC. Campgrounds, the pit toilet, and Redrock Falls (star) are designated.

Sampling Design

Sediment samples were taken approximately every 700m, with exact location marked with a GPS unit. At each site, an area approximately ¼ length across the river was isolated from the stream current using a bottomless bucket, a

sterile pipet tip dug 3 cm into the sediment and 1 mL of sediment and water drawn up. This was added to 1 mL of Amies transport media that discluded Sodium Thioglicollate. Biofilm samples were taken where a visible biofilm was present by scraping with the sterile collection tube and added to 1 mL of Amies media. Samples were packed on ice for transport and flash frozen in dry ice at the end of each collection day. Temperature readings were taken at each collection site, and observable oxygen content noted. Samples were specifically taken approximately 250 m before and after a point source. Sample days all were free of precipitation, with evening thunderstorms the day before.

DNA Extraction

All samples were vacuum filtered using a Buchner funnel and a 0.45 μm cellulose nitrate membrane. DNA was extracted from the membrane following the QIAGEN DNAeasy tissue extraction protocol, with the following modifications: samples were eluted into 1.8 mL Eppindorf tubes using a DNAeasy Mini Spin Column rather than a 96 well plate, and 2 elution steps were performed with 200 μL AE elution buffer per spin to increase DNA yield. DNA was extracted from an *Escherichia coli* culture of known titter using the same methods except that the solution was incubated at 56°C for four hours as opposed to overnight. All equipment was disposable plastic and guaranteed by the manufacturer to be sterile, DNase and RNase free.

Targeted Species	Primer Name	Primer Sequence (5'-3')	Tm	Product Size
Pirellula staleyi ATCC 27377	AP Forward Primer	AACACCAGTGGCGAA	59.61	83 bp
Hyphomicrobium denitrificans	AP Reverse Primer	GTATCTAATCCTGTTTGCTC	60.81	83 bp
Rhodoferax spp.	AR Forward Primer	CGGYAGAGGGGGATGG	62.18	171 bp
Aquabacterium commune str. B8	AR Reverse Primer	AGTTGACATCGTTTAGGG	60.4	171 bp
Chamaesiphon subglobosus PCC 7430	NC Forward Primer	CCWGTAGTCCTAGCCGT	60.16	86 bp
Nostoc GSV224 str. GSV224	NC Reverse Primer	CTAACGCGTTAAGTATCC	60.4	86 bp
Xanthomonas hyacinthi LMG 739	XN Forward Primer	GAAATGCGTAGAGATCGGG	62.57	143 bp
Xanthomonas melonis LMG 8670	XN Reverse Primer	TAAACGATGCGAACTGGAYGT	62.77	143 bp
Escherichia coli O157:H7	*stx*1 Forward Primer	GACTGCAAAGACGTATGTAGATTCG	60.02	150 bp
Escherichia coli O157:H7	*stx*1 Reverse Primer	ATCTATCCCTCTGACATCAACTGC	59.47	150 bp

Table 1. Primers used for RT-qPCR and their targeted species, Tm and amplicon size.

Real-Time Quantitative PCR

The group specific primers listed above were used to amplify specific species that are known to be in high abundance in Western Montana streams (Feris et al. 2003), plus an *Escherichia coli* assay to determine coliform distribution. A standard curve was created to determine absolute quantification of *E. coli* in each sample by serially diluting the eluted DNA from the culture of known titer by a factor of 10^{-2} for six standards. A standard was created for all the other samples by pooling 10 µL from each sample for the upper standard and diluting by a factor of 4 to make 6 standards total. (http://www.genetics.ucla.edu/labs/lusis/greenquantitative.htm).

2 replicas of each sample were included on each 96-well plate, and 2 plates were run for each primer pair. Each well contained 10 µL of QIAGEN SYBR Green-Based Master Mix, 0.2 µM of the forward primer and 0.2 µM of the reverse primer for the particular genera, 5 µL RNase-free water, and 1 µL of the sample. Samples were run using the Applied Biosystems 7500 RT-qPCR technology.

The following conditions were used in the reactions for each of the defined groups: *Pirellula* and *Hyphomicrobium* (AP), 50°C for 20 min, 95°C for 5 min, 45 cycles of 95°C for 15 s, 58.4°C for 30 s, 72°C for 1 min; *Aquabacterium* and *Rhodoferax* (AR), 50°C for 2 min, 95°C for 5 min, 45 cycles of 95°C for 15 s, 59.4°C for 30 s, 72°C for 60 s; *Nostoc* and *Chamaesiphon* (NC), 50°C for 2 min, 95°C for 5 min, 40 cycles of 95°C for 15 s, 57.3°C for 30 s, 72°C for 60 s; *Xanthomonas* (XN), 50°C for 2 min, 95°C for 5 min, 40 cycles of 15 s, 61.5°C for 30 s, 72°C for 60 s; *Escherichia coli* (stx1), 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 20 s, 55°C for 30 s, 62°C for 50s. The *E. coli* assay was run according to the Environmental Protection Agency (EPA) standards, with the exception of a slightly elevated extension temperature and time (Spano et al. 2005).

Statistical Analysis of Data

Data was analyzed first by performing an F-test on each of the series of 4 RT-qPCR quantities obtained for each sample for each primer pair in comparison to the top samples taken on each stream. An F-test was performed on *Escherichia coli* quantities between every sample and the sample above it on the stream, and on other species where large changes in concentration were seen. Each creek was

divided into above the point source and below the point source and an analysis of varience (ANOVA) was performed for each primer pair comparing above and below the point source levels. For Swift Current Creek, analysis was performed separately for both of the point sources considered. Analysis was performed on biofilm and sediment samples separately and then all sample types for each creek grouped. Every creek was analyzed individually, and then the control creek was compared to the two high-impact creeks as a group. For the control creek, a random point was chosen for above and below point source analysis for sediment samples in the same approximate location along the stream continuum as the point sources in the other streams. The biofilm sample taken in the control creek acted as the above point source sample, along with the biofilm taken above the point source in Swift Current Creek. All confidence levels were kept at 95%.

RESULTS

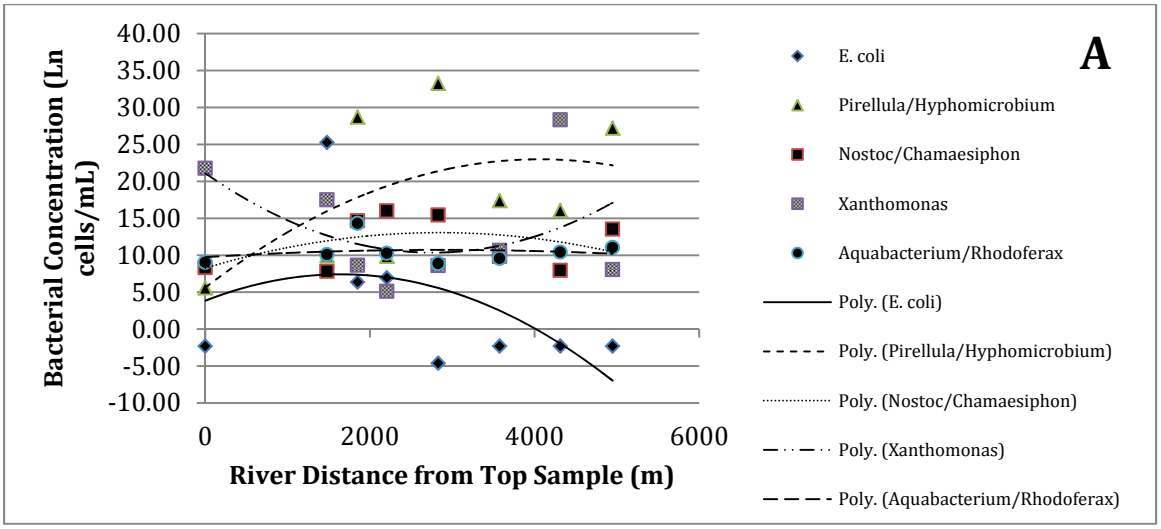
In Avalanche Creek, a significant increase in *Escherichia coli* in the sediment samples was found shortly below the location of the pit toilet in comparison to the sample taken above the point source ($p = 0.000$). This increase in *E. coli* drops back off to normal levels approximately 1500 m below the point source, yielding insignificance in the ANOVA for all samples above and below the pit toilet ($F = 0.302$, $p = 0.601$). *Pirellula* and *Hyphomicrobium* have great variance throughout the stream and show no statistical difference above and below the point source ($F = 2.14$, $p = 0.192$). *Nostoc* and *Chamaesiphon* exhibited a slight increase after the point source, but overall showed no significant trends ($F = 1.03$, $p = 0.348$). *Xanthomonas*

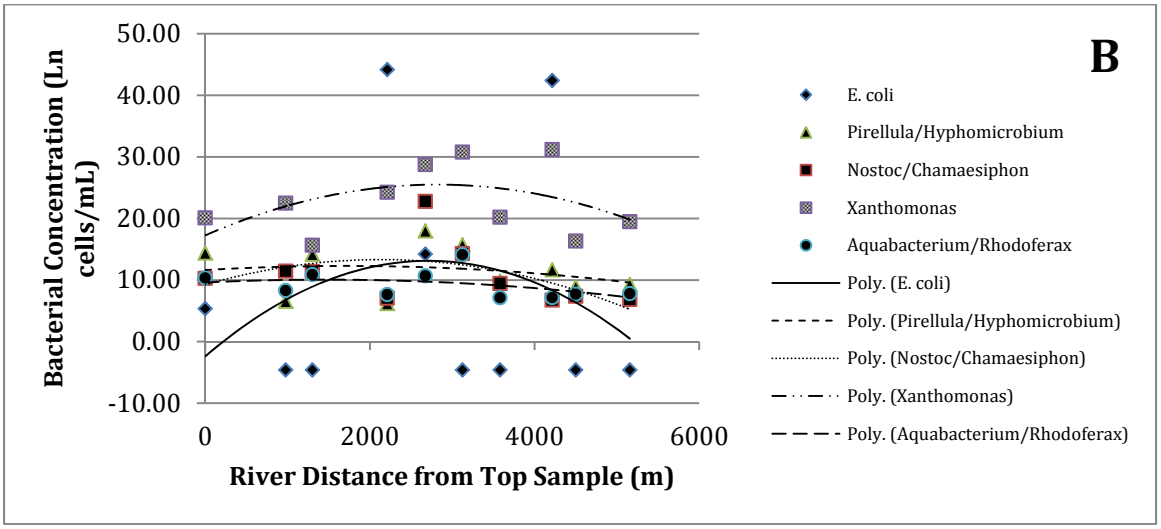
exhibits a significant decrease between the samples (p = 0.000) after the point source, but the levels return to normal further down the stream and there is no significant difference in the ANOVA (F = 1.19, p = 0.316). *Aquabacterium* and *Rhodoferax* have very little variance throughout the entire stream and no significant changes (F = 0.743, p = 0.422).

Escherichia coli levels in sediment in Swift Current Creek showed significant increases between samples at the lake (p = 0.000) and after the campground (p = 0.000), but dissipated further down the stream. The ANOVA found no statistical differences in *E. coli* for all samples above and below the point sources (F_{lake} = 0.015, p_{lake} = 0.902; $F_{\text{campground}}$ = 0.578, $p_{\text{campground}}$ = 0.502). For all other species, no statistical difference was seen above and below the lake point source or the campground point source for sediment samples only. When comparing sediment and biofilm samples together, a significant decrease was seen in *Nostoc* and *Chamaesiphon* after the campground (F = 7.740, p = 0.014).

The sediment samples in Valentine Creek saw no significant differences in *Escherichia coli* between all samples and above/below the randomly selected point source (F = 0.578, p = 0.502). There was no significant change in *Pirellula/Hyphomicrobium* (F = 0.594, p = 0.463), *Xanthomonas* (F = 0.041, p = 0.845), or *Aquabacterium/Rhodoferax* (F = 2.585, p = 0.147). A slightly significant decrease between all of the samples on Valentine Creek, with the exception of the final sample, was seen in *Nostoc* and *Chamaesiphon*, and slight significance was exhibited in the above/below point source analysis (F = 8.154, p = 0.065).

In comparing the sediment samples of both of the high-impact creeks (AC and SC) grouped together in comparison to the control (VC), significance was only found in concentrations of *Xanthomonas* above and below the pit toilet in Avalanche and the campground in Swift Current (F = 4.671, p = 0.462). Significance was also seen in the combined sediment and biofilm analysis of the grouped high-impact creeks for *Xanthomonas* below the campground on Swift Current (F = 5.586, p = 0.026).





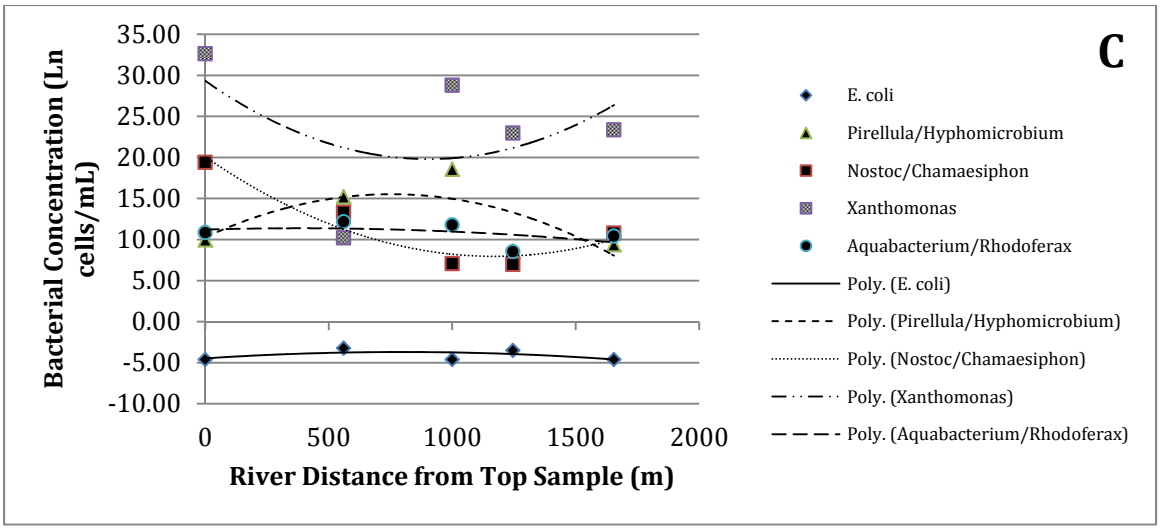


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Fig. 2. Bacterial concentration per sediment sample versus location along the stream continuum from the approximate headwaters to the confluence. A) AC: high human impact with a pit toilet at 1500 m; B) SC: high human impact with a heavy-use lake at 2200 m and campground at 4200 m; C) VC: no human impact.

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196 Analysis of the biofilm samples exhibited no statistical difference in
197 *Escherichia coli* (F = 0.205, p = 0.662), *Pirellula/Hyphomicrobium* (F = 0.443, p =
198 0.522), *Nostoc/Chamaesiphon* (F = 1.64, p = 0.234), and *Aquabacterium/Rhodoferax*
199 (F = 0.082, p = 0.781) levels above and below the point source. A slight increase in
200 *Pirellula/Hyphomicrobium* and a slight decrease in *Nostoc/Chamaesiphon* were seen
201 below the point source, but due to the variance of the samples, statistical
202 significance cannot be shown. There is a slightly significant decrease in
203 *Xanthomonas* below the point source (F = 3.744, p = 0.085).

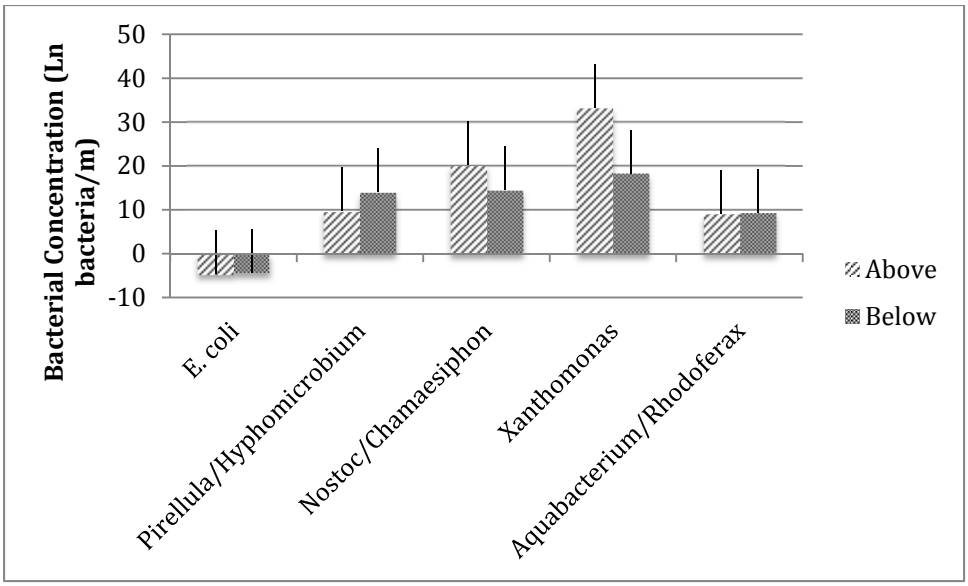

Fig. 3. Bacterial concentration in biofilm samples above and below the point source analyzed for all creeks combined.

DISCUSSION

Large peaks in *Escherichia coli* abundance are seen after every point source. No similar type of peaks are seen anywhere in the high-impact or control creeks. However, the *E. coli* levels drop back down to normal within 1500 m beyond the point source. This suggests that coliforms in the stream are in fact transient, and abundances to not build down the stream continuum. While coliforms are used in water testing due to their more hardy nature than many water pathogens, they do not appear to survive long enough to spread beyond the 1500 m limit.

The strain of *Escherichia coli* that was targeted in this study is the extremely pathogenic O157:H7. There are many other non-pathogenic strains of *E. coli* found in streams, but this strain was chosen due to its use by the EPA for water quality analysis and detection of fecal contamination (Spano et al. 2005). There is also an established RT-qPCR protocol for this strain. However, this strain does not contain

the genes to form a biofilm, and therefore it was not expected to see any differences in *E. coli* levels in the biofilms above and below the point source, nor was it expected to see high levels of *E. coli* in either. This limited the study in that *E. coli* differences in biofilms could not be detected and any correlation between the levels of *E. coli* and other species in the biofilms could not be drawn.

In both the biofilm and sediment samples, *Xanthomonas* was found to be the most affected by human pollution. This effect was seen at both levels: large decreases between individual samples and a large decrease in the overall above and below point source analysis. This change was especially apparent when comparing both of the high-impact streams grouped against the control stream. Members of the *Xanthomonas* genus are common plant pathogens and also produce a large amount of extracellular polysaccharides that could serve as the backbone for the establishment of biofilms. A decrease in *Xanthomonas* could cause an outgrowth of photoautotrophs in the stream due to the absence of this pathogen, or it could cause a decrease in healthy biofilm formation due to the lack of polysaccharides to form the matrix.

The nitrogen fixers, *Nostoc* and *Chamaesiphon*, appear to decrease down the stream continuum in the control creek. This trend supports the conclusions of previous studies that a greater concentration of nutrient cyclers is necessary higher in the stream due to the variable nature of nutrient influx at high elevations (Battin, et al. 2000). This trend is not seen in either of the high-impact creeks; the levels of the nitrogen fixers in these creeks stays relatively the same except after the campground on Swift Current Creek, indicating that the variability in nitrogen flux

and the need for nitrogen fixers may stay large throughout the high-impact stream continuum. It is expected that with eutrophication, nitrogen fixers will decrease in abundance due to the lack of ecological need for additional fixed nitrogen and the high-energy nature of the reaction. A slight decrease in nitrogen fixers is seen in the biofilms below the point source, but it is not significant. Correlation cannot be drawn between the presence of pollution and an effect on the nitrogen fixers because the results are very inconsistent and the difference between a natural process and pollution decreasing their abundance cannot be teased apart.

Differences above and below the point source were not seen in the other two bacterial groups. *Hyphomicrobium* is a nitrogen reducer and *Pirellula* is a sulfur scavenger, so it would be expected that an increase in these bacteria would exist in areas of eutrophication. A slight increase was seen in the biofilms, but this was not significant, and no differences were seen in the sediment samples. *Aquabacterium* is a member of healthy biofilms, so the lack of change above and below point source in all streams indicates that biofilms may not be as highly affected as hypothesized. *Rhodoferax* is a unique bacteria that can utilize iron-sugars in its metabolism; a change in this bacteria was not expected as iron-bound sugars levels should not change in the presence of human pollution. It is possible that any subtle changes in *Aquabacterium* are being overshadowed by the lack of change in *Rhodoferax*.

While *Escherchia coli* levels do significantly change with human pollution and presence, an affect is not seen for every species and the variance in species abundance could be due to other factors, such as chemical influx from the pollution or natural processes. This is especially the case when considering the spike in *E. coli*

seen at the lake, with no significant differences in the other species. This also suggests that human presence by itself it not having an effect on the bacterial biodiversity, as there were no known fecal influxes at this site.

Leaching from pit toilets and septic tanks in campgrounds appears to have a direct effect on the levels of pathogenic *Escherichia coli* in the stream. It also appears to have an effect on certain genera, particularly *Xanthomonas*. The genera targeted in this study were found to be in high abundance and stability in similar stream in Western Montana (Feris et al. 2003), but less abundant species that may be more fragile have not been studied and may be experiencing a greater effect. These species could be keystone species that are affecting the overall stream system, but could not be studied within the scope of this project because they are unknown and highly dependent upon other factors for which cannot be accounted.

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