A rural watershed containing a protected forest area in the north and a small rural community in the south was monitored for numbers of fecal indicator bacteria as well as the incidence and origin of the enteric pathogen *Salmonella*. Isolation of *Salmonella* only occurred once in the protected watershed, while downstream the isolation rate by the elevated-temperature swab technique ranged from 75 to 100%. This increase paralleled increases in fecal coliform and fecal streptococcus counts. A sheep herd grazing adjacent to the creek had a carrier rate for *S. arizonae* of 38.6%. However, this *S. arizonae* serotype was only isolated from Oak Creek on one occasion. *Salmonella* *bebe* was the most common serotype found in the creek and in surface runoff ditches in and around the rural community which flow into the creek. *Salmonella* MPNs downstream from the community ranged from 0.3 to 14 *Salmonella*/liter. These pathogens showed no injury, and persisted longer in this environment than fecal coliforms during
survival experiments using membrane diffusion chambers. The degree of persistence of *Salmonella* in diffusion chambers was inversely related to temperature. It was concluded from measurements of fecal indicator bacteria numbers and incidence and serotypes of salmonellae isolated that the bacteriological quality of this watershed was significantly affected by non-point runoff from the rural community. Domestic grazing animals in the area contributed only a nominal amount of salmonellae to the watershed.
Origin, Incidence, and Survival of $\text{Salmonella}$ in a Rural Watershed

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# TABLE OF CONTENTS

I. Introduction .............................................. 1
II. Materials and Methods ................................. 5
III. Results .................................................. 11
IV. Discussion .............................................. 18
V. Figures ................................................... 26
VI. Tables ................................................... 36
VII. Literature Cited ....................................... 40
VIII. Appendix A: Survival of *Salmonella* in Sheep Feces .............................. 47
IX. Appendix B: Survival of *Salmonella* in Water Stored in Redwood Tanks ............... 50
LIST OF FIGURES AND TABLES

Figure | Page
--- | ---
1 | Map of the Study Area | 26
2 | Map of the Community Area | 27
3 | Relationship Between Indicator Bacteria Levels, *Salmonella* Isolation Rates, and Oak Creek Site Number | 28
4 | Survival of Five Environmental *Salmonella* Isolates | 29
5 | Effect of Temperature on *S. give* | 30
6 | Comparison of Survival of Indicator Bacteria to Survival of *Salmonellae* | 31
7 | Comparison of Injury to Indicator Bacteria and *Salmonellae* due to Aquatic Exposure | 32
8 | Effect of Cold Shock and Medium of Growth on *S. give* Survival | 33
9 | Effect of Cold Shock and Medium of Growth on *S. give* Resistance to Aquatic Stress | 34
10 | Comparison of Survival of Environmental and Clinical *Salmonella* Isolates | 35

Table | Page
--- | ---
1 | Recovery of *Salmonella* from Oak Creek Sampling Stations | 36
2 | Recovery of *Salmonella* from Runoff Ditches in and Around the Rural Community | 37
3 | Oak Creek *Salmonella* MPN at Site 3: April, 1978, to April, 1979 | 38
4 | Oak Creek Water Characteristics Measured During Survival Experiments | 39
Origin, Incidence, and Survival of Salmonella in a Rural Watershed

INTRODUCTION

Section 208 of the Federal Water Pollution Act Amendments of 1972 (PL 92-500) directed individual states to identify and control non-point sources of water pollution, including agricultural and silvicultural sources. Much work has been done in both these areas, but little attention has been focused on other areas, such as the type and degree of effect of such potential sources as rural homes and communities. Sewage and septic effluent from a community can contain enteric pathogens (14,25,36,40). Therefore, monitoring for organisms such as Salmonella may provide more accurate information about surface water quality than simply measuring levels of fecal indicator bacteria, as there is no strict relationship between fecal indicator levels and Salmonella isolation (14,36,38). "Correlations" usually drawn simply state that above and below given fecal coliform densities, there are certain high or low chances, respectively, of Salmonella isolation (7,19,44).

One previous study was conducted to determine the effects of failing septic systems on a watershed (35). That study, which monitored only total and fecal coliforms, concluded that failing septic systems can degrade the bacteriological quality of a watershed. Although it was stated that 83% of the soils in the area were marginal to unsuitable for septic tanks, no comment was made regarding the age or general condition of these septic systems. The present study was
conducted in a watershed containing 78 homes, most of which were ten years old or less, served by individual septic systems, and which were constructed according to modern zoning criteria. The study area was well suited to this type of study since the creek had low fecal indicator bacteria levels as it emanated from the protected area. Therefore, any increases in indicator levels would be easily detectable.

Salmonella isolations from water have traditionally been associated with highly polluted water. With improved isolation techniques, however, isolations from water with low numbers of indicator organisms have been reported (9,11,12,14,38,40). Salmonellae have been detected in the absence of fecal coliforms (10), and there are cases of Salmonella in drinking water with few or no detectable coliforms (6,37). The sources of Salmonella in some unpolluted surface waters (less than 100 fecal coliforms/100 ml) is unclear (9,12). Their source in other water supplies has been attributed to sewage effluent (36,40), agricultural or urban runoff (10), or feedlot runoff (11). Aquatic Salmonella isolation has been associated once with accidental discharges from failing septic systems (14). In one study, rural homes were suspected to be the source of Salmonella in a watershed, but this was not proven (11).

The significance of Salmonella in the environment is compounded by the persistence of this organism. Gallagher and Spino (14) reported the isolation of Salmonella in a river 62 miles (99.8 km) downstream from a sewage outfall even though total and fecal coliform numbers had decreased by 99%. Salmonella tend to concentrate in bottom
sediments (25,44) as do fecal coliforms, and so lack of detection of salmonellae in overlying waters does not preclude their presence.

Andre et al. (1) reported that Salmonella survived about 16 d in farm pond water. Some investigators have reported Salmonella to survive about as well as fecal coliforms in the aquatic environment (27,38), while others (14) have reported Salmonella to persist longer than fecal coliforms, or not to persist as well as fecal coliforms initially (18). McFeters and Stuart (28) observed a great difference in E. coli survival between two creeks, and later McFeters et al. noted that conclusions regarding survival must be approached with great caution due to the multitude of uncontrollable parameters in natural waters (27). These authors and others (13,18) have concluded that temperature is very important, if not the most important determining factor for E. coli persistence, and that persistence times are inversely related to temperature.

Persistence of bacteria in the environment is a function of susceptibility of organisms to stresses encountered in their specific environment, and consequent injury. Induced injury and subsequent repair has been demonstrated in Salmonella and other gram-negative bacteria (21,22,32,33,39,43). Fecal coliforms (4,5,41) and fecal streptococci (4) have been shown to become stressed during aquatic exposure as demonstrated by differential recovery on selective and nonselective media. This same phenomenon has been observed in Shigella in sewage (45). Recently, McFeters et al. (29) have reported aquatic injury to S. typhimurium including increased deoxycholate sensitivity.
The purpose of the present study is to quantitate non-point pollution on a rural watershed containing a community of defined size and a known number of domestic grazing animals. It is believed that the defined nature of the study basin and the measurement of both fecal indicator bacteria densities and *Salmonella* incidence throughout the study area makes this study the first of its type. The tributary stream model was chosen since virtually no water quality data are available on Oregon tributary streams in headwater areas; most sampling sites are on principal waterways (51).
MATERIALS AND METHODS

Study Area

The Oak Creek watershed (Figure 1) is a rural watershed approximately 11 km northwest of Corvallis, Oregon. The watershed covers a 750 ha area. The upper (northern) part of the basin drains part of McDonald State Forest, a 4450 ha forest which is undisturbed except for research activities. The southern border of the forest is midway between sites 7 and 8, running east-west. The lower (southern) part of the basin includes a rural community consisting of 78 homes, most of which are less than 10 years old, served by individual septic tank systems. The community is most dense about the west side of the site 7 area (Figure 2). The average annual rainfall is 102-105 cm; measurable precipitation occurs on the average of 150-160 d/year. Seventy percent of the precipitation falls between November and March. The frost-free season is 165-200 d/year.

Above site 2 the creek runs through soil of the Dixonville-Philomath association, which consists of moderately deep, well-drained silty clay loams in this area. Below site 2, the basin consists of soils of the Waldo-Bashaw association, which consists of poorly drained silty clay loams and clays. The slope of the creek in these areas is 0-3%, and runoff is slow or ponded. The seasonal high water table is 0-15 cm. These factors place severe restrictions on the use of septic tank absorption fields. Sites 1 and 3-6 are closely bounded by soils of the fine, mixed, mesic family of Fluvaquatic Haplaquolls.
The slopes vary from 0-3%; runoff is slow with only slight erosion. Because of slow soil permeability and peak water tables near the surface during the rainy season, the area is unsuitable for septic tank use. Sites 7, 8, and 9 are bordered by soils of the fine, mixed, mesic Pachic Ultic Argixerolls. The community is built on slopes of 3-12%. Runoff is medium to rapid, and slopes bordering the creek vary from 3-5%. The water table is fairly deep, but because of slow soil permeability and shallow bedrock, the area is considered marginal to unsuitable for septic tanks. East of site 3, and bordering site 10, the soil is of the clayey, montmorillonitic, mesic, shallow family of Vertic Haploxerolls. Slopes vary from 3-45%, and runoff is medium to rapid. The water table is not shallower than 150 cm, but the soil is shallow, and there is a high shrink-swell potential.

**Bacterial Cultures**

*Salmonella typhimurium* LT-2, *S. enteritidis* ser. Paratyphi B, and *S. typhi* were obtained from the stock culture collection of the Department of Microbiology, Oregon State University. *Escherichia coli* SML-3 was isolated from raw sewage from the Corvallis, Oregon Wastewater Treatment Plant. *Streptococcus faecalis* OSU-23 was isolated from Oak Creek. The latter two organisms were identified according to Standard Methods (2).
Isolation Media and Methods

Pfizer's Selective Enterococcus Agar (PSE) was obtained from Gibco Diagnostics (Madison, Wisconsin). All other dehydrated media were obtained from Difco. Isolation of Salmonella from water was done by Moore swabs (30). Swabs were placed in Oak Creek for 3 days or in runoff ditches surrounding the community for 7 days. Retrieved swabs were returned to the laboratory within 20 min, and placed into 250 ml tetrathionate broth (Tet), supplemented with 10 mg/liter brilliant green (16), and incubated at 41.5°C for 48 h. After incubation, Tet was streaked to brilliant green (BG) and bismuth sulfite (BS) agars, which were incubated at 35°C for 24 h. Brilliant green agar was supplemented with 8 mg/liter sulfadiazine (15). Presumptive colonies were streaked for isolation on MacConkey agar (35°C for 24 h), then inoculated into triple sugar iron agar and lysine iron agar slants (35°C for 24 h). API 20E test strips (Analytab, Plainview, New York) were used to biochemically identify representative isolates. Presumptive isolates were confirmed as Salmonella using pooled antisera (Difco). Definitive serotyping was performed by the State of Oregon Department of Agriculture. Salmonella were isolated from other specimens by placing 25 g of feces, sheep feed, or soil, or 10 g of litter (leaves, grass, and dry vegetation) into 250 ml Tet, and incubating at 41.5°C for 48 h. Salmonella were identified as previously indicated.

Salmonella were enumerated from water and fecal samples using an MPN technique. Five replicate samples each of 1,000 ml, 100 ml, and
10 ml of Oak Creek water were filtered through 0.45 μm membrane filters (Gelman #GN-6), which were placed into 100 ml Tet, and incubated at 41.5°C for 48 h. *Salmonella* were isolated and identified as previously indicated.

Standard Plate Count Bacteria were enumerated by the Standard Methods technique (2). Total and fecal coliforms were enumerated using the Standard Methods membrane filtration technique. Fecal streptococci were enumerated by membrane filtration using PSE agar. Membrane filters were obtained from Gelman (0.45 μm, #GN-6).

The broth medium for cultivation of bacteria to be used in survival experiments was designated PCB, and except for agar, contained the ingredients present in Standard Plate Count Agar. PCB cultures were incubated with shaking at 35°C for 18 h. TSY broth consisted of tryptic soy broth plus 0.3% yeast extract and 0.5% glucose (28). TSY cultures were also incubated with shaking at 35°C for 24 h. TSY agar consisted of TSY broth plus 1.5% agar. Tet agar consisted of Tet plus 1.5% agar, steamed for 30 min, and cooled to 45°C. The iodine/potassium iodide supplement was then added and plates poured. Care was taken to keep the calcium carbonate component of Tet in suspension by frequent magnetic stirring while the plates were being poured. Standard Plate Count Agar (PCA) incubated at 35°C for 24 h was the standard recovery medium for survival experiments. BG, BS, TSY, PSE, MacConkey and Tet agars were incubated at 35°C for 24 h. mFC agar was held at both 35 and 44.5°C for 24 h.
Survival Experiments

Membrane diffusion chambers (MSU-VME Diffusion Chambers, Valley Machine Inc., Belgrade, Montana) were used to study the in situ survival of various pathogens and indicator bacteria in Oak Creek. Chambers were fitted with 0.2 μm Gelman Acropor AN-200 filters, and autoclaved for 15 min at 121°C with filters in place. After growth in PCB, five ml of culture was added to 25 ml sterile Oak Creek water (SOC) (27), centrifuged, washed twice in SOC, and diluted to approximately $10^6$ cells/ml with SOC. One hundred ml of cell suspension was added to each sterile chamber. Chambers were then immediately transported to the experimental site (site 3) in refrigerated Oak Creek water. Transport temperature was 4°C in winter and 12°C in summer. Chambers were equilibrated for 1 h in Oak Creek before the initial sampling. One ml aliquots from chambers were placed into 9.0 ml dilution blanks, which consisted of Standard Methods phosphate buffer plus 0.1% peptone (2), and immediately transported back to the laboratory (about 10 min). Five replicate spread plates of the appropriate dilution were prepared for each recovery medium.

To determine whether cold shock affected Salmonella survival, and the influence of medium of growth on the cold shock effect, the following experiment was designed. 18 h, 35°C, shaking broth cultures of an S. give isolate designated S6 were prepared as follows. Treatment 1: grown in TSY broth, then washed and centrifuged as described. Treatment 2: grown in PCB, then washed and centrifuged as described. Treatment 3: grown in TSY, cold shocked by pipetting 5 ml
of culture into 25 ml of 4°C SOC on ice, then performing all centrifugation and dilution steps at 4°C. Treatment 4: grown in PCB and cold shocked. The four differently treated suspensions of S. give were then placed into chambers, which were placed in Oak Creek and sampled daily for 6 d by plating dilutions on PCA, TSY, BG and BS agars.

Various chemical properties of Oak Creek water were measured during the course of survival experiments in order to determine any correlation(s) between water characteristics and bacterial survival, and to insure that Oak Creek water did not have any "unusual" characteristics which would make it atypical and unrepresentative of a rural watershed. Temperature was measured daily with a Taylor No. 5458 Maximum-Minimum Self-Registering Thermometer (Taylor Instrument Consumer Products Division, Arden, North Carolina). Conductivity was measured at least once during every survival experiment with a YSI Conductivity Bridge, Model 31, fitted with a YSI 3402 probe (Yellow Springs Instrument Co., Yellow Springs, Ohio). Dissolved oxygen was measured once every survival experiment with a YSI Model 51B Oxygen Meter, fitted with a YSI 5739 probe. pH, nitrate, and calcium were measured once every survival experiment with an Orion Ionalyzer Model 407A.
RESULTS

To more clearly define the non-point source(s) of fecal pollution entering Oak Creek, a study of the presence and origin of the enteric pathogen Salmonella in the Oak Creek watershed was conducted simultaneously with a survey of fecal indicator bacteria densities in the creek. This was done to ascertain whether specific levels of fecal pollution entering the creek could be associated with specific sources by common Salmonella serotypes.

From December, 1977 to June, 1979, the Oak Creek watershed (Figure 1, Figure 2) was surveyed for the presence, kinds, and density of Salmonella (Table 1, Table 2). Salmonella give was found to be the most common serotype in the creek and in surface runoff drainage ditches (82.7% of all creek isolations and 66.7% of all ditch isolations). Salmonella bareilly and three serotypes of S. arizonae were also isolated from the creek. Each S. arizonae serotype agglutinated without cross-reaction in Difco polyvalent antisera. The serotype designated S. arizonae C reacted with Salmonella O Antiserum Poly C; S. arizonae D reacted with Poly D; and S. arizonae G reacted with Poly G. Salmonella arizonae C was isolated once at site 8, which was the only Salmonella isolation north of site 7 (Figure 1). South of site 8, isolations were increasingly frequent as the creek flowed into the populated region of the study basin. As seen in Figure 3, this increase in Salmonella isolation rate closely paralleled the increase in fecal coliform and fecal streptococcus...
densities, and roughly paralleled the increase in total coliform counts. As measured by both Salmonella incidence and fecal indicator densities, the vast majority of the bacteriological pollution entering the creek was found around site 7 (Figure 2). Inspection of average cell densities of fecal coliforms and fecal streptococci illustrates that counts at site 3 were one to two logs higher than those at site 9 (Figure 3). Sites 2 and 1 are 1.1 and 3.8 km downstream, respectively, from site 3, and contain significantly lower cell densities than site 3. The area between these sites is thinly populated and would receive little septic runoff (Figure 1).

A potential source of fecal pollution in the creek, 100 head of sheep are pastured adjacent to the creek between sites 3 and 7 for 90 d/year. Geldreich (17) has estimated that 1.13 kg wet weight of feces are excreted per sheep per day. Based on this estimate, slightly more than $10^4$ kg feces/year are excreted by these sheep during the pasture period. Since between 3 and 15% of sheep are Salmonella carriers (19), a survey of pasture litter and soil, and sheep feces was done. Twenty-two of 57 (38.6%) of sheep feces were positive for S. arizonae G. No other Salmonella serotypes were isolated from the feces. Nine soil samples and four litter samples from the sheep pasture were negative for Salmonella. Examinations confirmed that the source of S. arizonae G in sheep feces was Mexican bone meal feed. Rolled barley, alfalfa pellets, and salt/phosphate mixture were all negative for Salmonella. Despite the high carrier rate in the sheep herd, S. arizonae G was only isolated from
Oak Creek on one sampling date, from sites 2, 5, 7, and 12. This low isolation incidence may be at least partially attributable to the low numbers of *S. arizonae* in the sheep feces. Two *Salmonella*/100 g feces was determined to be the MPN density for carrier sheep. Another potential source of fecal pollution in the creek, a horse corral adjacent to site 7, was also sampled. Ten horse feces samples collected from the corral were negative for *Salmonella*.

Because of the high qualitative isolation rate of salmonellae from the creek south of site 7, *Salmonella* numbers were quantified at site 3. This was done to determine what *Salmonella* density corresponded to the higher isolation rates. MPN determinations were done from April, 1978 to April, 1979 (Table 3). Despite the high qualitative isolation rate at site 3, *Salmonella* cell densities were very low, ranging from <0.3 to 14 salmonellae/liter, with a mean of approximately 2 salmonellae/liter.

Some of the drainage ditches in the study area (Figure 2) which carry surface runoff from the community were surveyed for fecal coliform levels. Fecal coliform densities in ditch G ranged from a low of 60/100 ml during times of low flow and no rain to $4.4 \times 10^3$/100 ml following ground saturation with moisture and periods of heavy rain. Two other ditches monitored for fecal coliform levels showed similar changes. Ditch A ranged from a low flow level of 2 fecal coliforms/100 ml to a high of $4.8 \times 10^2$ fecal coliforms/100 ml during heavy rain. Similarly, ditch F had 50 fecal coliforms/100 ml during low flow, and $5.1 \times 10^3$ fecal coliforms/100 ml during heavy rain. Because of the
presence of fecal coliforms in these ditches, the presence of Salmonella was monitored to determine whether these ditches were the source of Salmonella in Oak Creek (Table 2). Salmonella give was isolated from all ditches with run-off conditions prevailing, except ditches B and C, from which no salmonellae were isolated. These two ditches are located downslope from the area of lowest housing density. Salmonella arizonae G was isolated once, from ditch A, concurrently with S. give. Salmonella give was isolated on every attempt from ditch A, both upslope and downslope from the sheep pasture. This ditch runs below several homesites upslope from the sheep pasture and site 3. In addition to S. give, which accounted for 66.7% of all ditch Salmonella isolations, S. nottingham and S. arizonae were also isolated from ditches. Salmonella nottingham was isolated once, from ditch F, but was not isolated from Oak Creek. Salmonella arizonae C was isolated once from ditch D, and three times from ditch G. Salmonella arizonae D was not isolated from any ditches but was isolated once from Oak Creek site 12. Salmonella bareilly, which was isolated once from Oak Creek, was not isolated from any ditches.

Survival Experiments

Having determined the origins of Salmonella in Oak Creek, experiments were conducted to quantitatively measure the survival of salmonellae with membrane diffusion chambers immersed in the creek at site 3. Additional studies compared the survival times of, and the influence of selective media on, fecal indicator bacteria and
salmonellae. Measurements of Oak Creek water characteristics were done during these experiments, and are tabulated in Table 4.

Survival of five Salmonella isolated from this watershed was compared (Figure 4). Although there was some difference in survival rates, all persisted well over 6d. The effect of temperature on the survival of an S. give isolate designated S6 is shown in Figure 5. Lower temperature enhances survival by extending the lag period before exponential dieoff begins. After 8 days with a 6-12°C in situ water temperature range, 40% of the organisms remained. In contrast, under a 13-21°C range, less than 1% of the organisms remained at the end of the same time period.

Additional survival experiments were performed in order to compare persistence characteristics of indicator bacteria with those of salmonellae. Salmonella give and S. arizonae G survival characteristics were compared to E. coli and S. faecalis. The persistence of (Figure 6), and injury to (Figure 7), these organisms is illustrated. In agreement with Bissonnette et al. (4), both indicators die off rapidly and show injury on their respective selective media. In contrast to these results, both salmonellae persisted well without detectable injury. After 72 h, recovery on PCA showed about 50% of the salmonellae remained, while only 1% of the indicators remained. On selective media, about 50% of the salmonellae were recovered, but only 0.1% of the indicators were recovered.

Lack of injury to these salmonellae following exposure to the aquatic environment was a surprising result. Recovery of the five
Salmonella isolates seen in Figure 4 was compared on PCA and BG agars (data not shown). There was no significant difference in recovery on the two media, again indicating lack of detectable injury to these salmonellae. After injury by freezing, Salmonella anatum and E. coli have shown sensitivity not seen in uninjured cells to surface active agents such as deoxycholate (33,34). Since mFC agar contains bile salts but BG and BS agars do not, recovery of Salmonella on bile salt-containing media was done to determine any inhibitory effects of bile salts on aquatically stressed Salmonella such as was observed for E. coli when comparing PCA and mFC agars. There was no significant difference in S. give recovery over a 6 d period on PCA, mFC, MacConkey, or Tet agars, or PCA containing 1.5 g/liter bile salts No. 3 (Difco) (data not shown).

An experiment was done to determine if susceptibility to aquatic stress and injury was induceable by cold shock. Salmonella give was grown in PCB and the nutritionally richer TSY broth. Survival of unshocked and cold shocked cells from each medium was compared (Figure 8). There was no apparent difference in survival due to cold shock in cells grown in either medium. As seen in Figure 9, cold shock not only had no effect on S. give survival, there was also no injury induced by cold shocking, in either medium.

Finally, determination of S. give survival in comparison to known clinical Salmonella isolates was studied to ascertain possible differences in survival characteristics between environmentally-derived and clinical isolates (Figure 10). Salmonella give survived slightly longer than
S. typhimurium and much longer than S. enteritidis ser. Paratyphi B or S. typhi. At 6 d, 5% of the S. give remained; only 0.6% of S. enteritidis ser. Paratyphi B, and 0.05% of S. typhi remained.

The range of Oak Creek water parameters is shown in Table 4. These parameters, which were measured during the course of the survival experiments, indicate that Oak Creek water was not atypical for water from a rural watershed.
DISCUSSION

The uniquely high increase in fecal indicator densities in the creek below the community clearly demonstrate that the community was a non-point source of pollution in this watershed. No other similar increase in bacterial pollution indicators was recorded in other portions of the watershed. Indeed, a decrease in pollution indicators downstream from site 3, where the population density is reduced, was noted. Reneau et al. (35) previously observed a decrease in fecal coliforms downstream from failing septic systems, and attributed the phenomenon to dilution, sedimentation, and bacterial dieoff. The presence of S. give in both the creek downstream from the community, and the runoff drainage ditches below the community further implicate the community as a source of non-point pollution. This presence of S. give also provides a solid epidemiological link between the community as a source of Salmonella and Salmonella in the creek.

Domestic grazing animals in the study area appeared to contribute a negligible amount of salmonellae to this watershed. Lack of Salmonella in feces from horses in the watershed and the single isolation of the sheep-associated S. arizonae G from the creek indicate that grazing animals have an insignificant impact on water quality as measured by the presence of Salmonella.

Even though fecal indicator counts for steady state high flow (winter) are ten-fold lower than steady state low flow (summer), (data not shown), salmonellae were isolated consistently, without seasonal variation. This is in contrast to previous findings of seasonal
variation in Salmonella isolation (8) where salmonellae were isolated much more often in the summer months than in the winter, and implies a constant reservoir in the watershed. One such reservoir of Salmonella may have been the stream sediment. Despite the great seasonal variation in indicator counts, and the consistently high Salmonella isolation rate downstream from the community, Salmonella densities in the creek remained consistently low. This apparent enigma may have been the result of the MPN Salmonella enumeration technique. Since MPNs were constantly in the low end of the detectable range, statistically significant fluctuations in Salmonella cell densities between sampling times may have gone undetected.

Previously reported Salmonella MPNs have been much higher than those reported here. Salmonella cell densities as high as 46/100 ml in an estuary (26), and as high as 54/100 ml in a river (38) have been reported. As in this study, Cherry et al. (9) found Salmonella numbers to be low in the surface waters studied (less than one Salmonella/100 ml), but recovery rates were good. The ability to isolate salmonellae from this creek by the swab technique virtually at will provides proof of the efficacy of the elevated temperature swab technique which was proposed by Spino (40), and is now a recommended technique in Standard Methods (2).

The inability to quantify with greater precision Salmonella cell numbers in this stream does not negate the significance of these findings. The fact that the community contributes Salmonella to the
creek as a result of non-point source runoff emphasizes the im-
portance of re-evaluating criteria for the installation of septic systems
in similar rural communities. More attention must be directed toward
selection of proper soil types and favorable slopes in order to assure
that rural developments do not become significant sources of non-
point pollution, which could have been prevented by proper considera-
tion of such factors. It is important to emphasize that the measure-
ment of only fecal indicators would have implicated the community as a
source of non-point pollution but detecting specific Salmonella sero-
types conclusively showed what fecal indicators only imply: the
presence of pathogens.

The greatest non-point pollution potential from septic failure
or overflow is manifested in winter. The reasons for this are two-
fold. First, the rainy winter season provides the maximum chance of
creek contamination by enteric organisms due to surface runoff from
septic failure, and subsurface movement under saturated flow conditions
(23). Second, the survival potential of Salmonella is greatly enhanced
by lower winter temperatures, as seen in Figure 5. Faust et al. (13)
showed a similar temperature effect on E. coli, and in addition
observed that the presence of montmorillonite greatly enhanced
survival time. The greater volume of suspended solids including fine
clays during high winter flow may similarly enhance the survival of
salmonellae in Oak Creek.

The significance of salmonellae in the environment is related to
their ability to persist. The longer persistence and apparently greater
resistance to stress than fecal indicator bacteria in the creek environ-
ment may account for the high Salmonella isolation rate concurrent with
decreasing numbers of fecal coliforms downstream from site 3. Considering the low Salmonella cell densities at site 3 (Table 3), it seems amazing that while fecal coliform densities from site 3 to site 1 would decrease by nearly two log values (Figure 3), the percent Salmonella isolation rate only decreased from 92.3% to 83.3%. Such persistence may help explain Gallagher and Spino's (14) isolation of Salmonella 99.8 km downstream from a sewage outfall, despite a 99% reduction in total and fecal coliforms. The isolation of Salmonella over a wide fecal coliform density range in this study confirms previous findings that even though fecal coliforms give a rough indication of Salmonella presence, there is no strict correlation between fecal coliform levels and Salmonella isolation (14,36,38).

Previous studies on persistence of bacteria in the environment in relation to aquatic injury have focused primarily on indicator organisms (4,5,40). Our results concur with Bissonnette et al. (4), who observed aquatic stress in E. coli and S. faecalis. In the present study, survival of (Figure 6), and injury to (Figure 7) these indicator bacteria was observed over a 72 h period. Injury in both organisms appeared to increase over time, as measured by recovery on selective vs. nonselective media. The injury to the fecal coliform-positive E. coli was obviously a medium effect, as recovery on mFC agar was identical at 35 and 44.5°C.

In contrast to the fecal indicator bacteria survival characteristics, both S. give and S. arizonae G showed a nominal decrease in cell viability and no injury over 3 d. Not only did these two salmonellae
show relatively little loss in viability over 3 d, but they showed no injury on recovery on BG or BS agars when compared to PCA. Over a 6 d period, none of the Salmonella isolated in this study showed any difference in recovery on BG and PCA agars.

Salmonellae have been reported to survive as well as, poorer than, and greater than, fecal coliforms (14,18,27). This inconsistent relationship in survival characteristics, and lack of correlation between fecal coliform levels and Salmonella isolation (14,36,38) coupled with the lack of injury to salmonellae in the aquatic environment observed here suggest that use of fecal coliforms may be inadequate indicators of the presence of pathogens. As a result of aquatic stress, 90% of the E. coli in the survival chamber were injured and therefore went undetected on mFC agar after 3 d exposure to Oak Creek. If the survival chamber model is an accurate representation of bacterial persistence in Oak Creek, a method of recovering both the injured and uninjured populations of fecal coliforms could have resulted in significantly higher levels of recovery than determined by mFC agar. Methods of enumerating both injured and uninjured populations of fecal coliforms from water (5,42) and frozen foods (40) have been proposed, but have not yet come into general use.

BG and BS agars contain the bacteriostat brilliant green, but contain no bile salts. In order to compare Salmonella sensitivity to bile salts with E. coli, S. give recovery on bile salts-containing media was compared to recovery on PCA. There was no difference in
numbers of salmonellae recovered, even on mFC agar, which failed to
recover 90% of \textit{E. coli} that had been exposed to the aquatic environ-
ment for 3 d. These results indicate a lack of sensitivity to bile salts
in aquatically exposed salmonellae that injured enteric organisms
demonstrate (33,34).

McFeters et al. (29) have recently reported observation of injury,
including increased sensitivity to deoxychloate, in \textit{S. typhimurium}
after exposure to either reagent grade water or an unpolluted creek.
While strain differences may account for variable sensitivities to bile
salts, it is possible that other factors may account for sensitivity
to aquatic stress. Greater persistence has been found in a creek with
higher conductivity and divalent cations (28) when bacterial survival
in two creeks was compared. Oak Creek has a conductivity (Table 4)
of at least ten times the creeks used in the latter study, and pre-
sumably even higher than the aquatic environments studied by McFeters,
et al. (30). The importance of divalent cations in maintaining cell
wall integrity in gram-negative organisms is well known (21,24,34). The
higher ionic content of Oak Creek may afford greater protection to
\textit{Salmonella} than the water sources McFeters et al. studied, and there-
fore prevented aquatic injury. No other parameter measured here appears
to have a similar protection effect on \textit{Salmonella} survival in the aquatic
environment. This does not account for the observation of injury in Oak
Creek to \textit{E. coli}. If Oak Creek water afforded protection from aquatic
stress to \textit{Salmonella}, then the cold shock treatment which was ineffective
here in inducing susceptibility to stress may have been effective if the
cold shocked salmonellae had subsequently been placed in an aquatic environment less conducive to repair.

It is possible that the source of salmonellae determines their survival potential. The greater survival potential of environmentally-derived salmonellae than known clinical isolates shown here implies that the environmental isolates were better adapted for survival in the aquatic environment. Even though the environmental Salmonella isolates persist better in the aquatic environment, their virulence may be no less than clinical isolates. It has previously been shown that there is no difference in virulence between clinical and environmental isolates of the opportunistic pathogen Klebsiella pneumoniae (3). Further, the lack of sensitivity to bile salts, even after aquatic exposure, seen in Salmonella here implies no virulence loss due to environmental exposure, as bile salts resistance in Salmonella has been correlated with virulence (42).

A previous study on Salmonella incidence in watersheds containing rural unsewered communities, small sewered urban areas, and a cattle feedlot was done in central New York state (11). The feedlot and the urban areas were the only definitely defined sources of Salmonella in the area. The unsewered communities were suspected as being sources of pollution in the study area, but this possibility was not investigated. Reneau et al (35) studied the increase in total and fecal coliforms in a watershed below failing septic systems, but did not attempt to isolate Salmonella. The present study is believed to be the first comprehensive study to incorporate fecal indicator bacterial
measurements and *Salmonella* surveillance to epidemiologically link a rural community to non-point pollution in a watershed.

The most likely source of *Salmonella* in this watershed, given the nature of the soil and extent of slopes in the area is the septic tanks of the community. On this assumption, recommendations may be made to prevent contamination of this type from polluting watersheds under like construction. Recommendations (20) proposed for similar situations where septic effluent surfaces in areas unsuitable for septic systems include (i) banning further construction in the area until the problem is mitigated, which may effectively ban any subsequent construction, and (ii) provide sewer facilities to the area, an expensive and difficult solution in this case. As an alternative which would prevent future non-point pollution of this type, we recommend a change in zoning laws to restrict installation of septic fields in similarly poor drained soil and/or areas where slopes are excessive, or enforcement of such regulations if they should exist.
Map of the Oak Creek watershed. Oak Creek sample sites are numbered. The southern boundary of McDonald State Forest runs east-west midway between sites 7 and 8.
The community is on the west side of Oak Creek, concentrated primarily about site 7. Oak Creek sample sites are numbered; ditches are lettered.
FIGURE 3 - RELATIONSHIP BETWEEN INDICATOR BACTERIA LEVELS, SALMONELLA ISOLATION RATES, AND OAK CREEK SITE NUMBER.

Stream flow runs downstream from site 9. Indicator bacteria levels were measured at peak high flow during rainfall with much surface runoff. Salmonella isolation rates over the 18-month study period, compared to a representative sampling of indicator bacteria counts. These counts, done in duplicate, represent average high flow indicator counts (minimum of five samplings) at each site.
Isolates were enumerated on five replicates of PCA agar following incubation at 35°C for 24 h. No significant difference in counts was observed when cultures were plated on PCA or BG agars. The serotype of *S. arizonae* S8 is G; the serotype of S29 is D; the serotype of S38 is C.
FIGURE 5 - EFFECT OF TEMPERATURE ON *S. give* SURVIVAL

![Graph showing the effect of temperature on *S. give* survival.](image)

*Salmonella give S6*

- **●** TEMPERATURE RANGE 6-12°C
- **△** TEMPERATURE RANGE 13-21°C
S. arizonae S8 was isolated from sheep feces; the serotype of this isolate is G.
FIGURE 7 - COMPARISON OF INJURY TO INDICATOR BACTERIA AND SALMONELLA DUE TO AQUATIC EXPOSURE
FIGURE 8 - EFFECT OF COLD SHOCK AND MEDIUM OF GROWTH ON S. 

Salmonella give S6

Treatment 1: TSY broth grown, gentle treatment.
Treatment 2: PCB grown, gentle treatment.
Treatment 3: TSY grown, cold shocked.
Treatment 4: PCB grown, cold shocked.
FIGURE 9 - EFFECT OF COLD SHOCK AND MEDIUM OF GROWTH ON S. *gass* RESISTANCE TO AQUATIC STRESS

Treatment 1: TSY broth grown, gentle treatment.
Treatment 2: PCB grown, gentle treatment.
Treatment 3: TSY grown, cold shocked.
Treatment 4: PCB grown, cold shocked.
FIGURE 10 - COMPARISON OF SURVIVAL OF ENVIRONMENTAL AND CLINICAL SALMONELLA ISOLATES
TABLE 1 - RECOVERY OF SALMONELLA FROM OAK CREEK SAMPLING STATIONS

<table>
<thead>
<tr>
<th>SITE</th>
<th># OF ISOLATIONS</th>
<th>% Incidence</th>
<th>SEROTYPES ISOLATED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># OF ATTEMPTS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5/6</td>
<td>83.3</td>
<td>give</td>
</tr>
<tr>
<td>2</td>
<td>3/4</td>
<td>75.0</td>
<td>give, arizonae G</td>
</tr>
<tr>
<td>3</td>
<td>12/13</td>
<td>92.3</td>
<td>give, bareilly</td>
</tr>
<tr>
<td>4c</td>
<td>7/8</td>
<td>87.5</td>
<td>give</td>
</tr>
<tr>
<td>5</td>
<td>7/8</td>
<td>87.5</td>
<td>give, arizonae G</td>
</tr>
<tr>
<td>6</td>
<td>2/2</td>
<td>100.0</td>
<td>give</td>
</tr>
<tr>
<td>7</td>
<td>3/6</td>
<td>50.0</td>
<td>give, arizonae G</td>
</tr>
<tr>
<td>8</td>
<td>1/7</td>
<td>14.3</td>
<td>arizonae C</td>
</tr>
<tr>
<td>9</td>
<td>0/8</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>10c</td>
<td>3/4</td>
<td>75.0</td>
<td>give, bareilly</td>
</tr>
<tr>
<td>12</td>
<td>6/8</td>
<td>75.0</td>
<td>give, bareilly, arizonae D, arizonae G</td>
</tr>
</tbody>
</table>

TOTAL 49/74 $\bar{x} = 66.2$

\(^a\text{Incidence} = (\# \text{ of isolations}/\# \text{ of attempts}) \times 100\%

\(^b\text{Three distinct S. arizonae serotypes were isolated from Oak Creek. Each S. arizonae serotype agglutinated without cross-reaction in Difco polyvalent antisera. The serotype designated S. arizonae C reacted with Salmonella O Antiserum Poly C; S. arizonae D reacted with Poly D; S. arizonae G reacted with Poly G.}

\(^c\text{Denotes sites in Skunk Creek.}\)
### TABLE 2 - RECOVERY OF SALMONELLA FROM RUNOFF DITCHES IN AND AROUND THE RURAL COMMUNITY

<table>
<thead>
<tr>
<th>SITE</th>
<th># OF ISOLATIONS</th>
<th>% INCIDENCE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SEROTYPES ISOLATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6/6</td>
<td>100.0</td>
<td>give, arizonae G&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>0/2</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>0/2</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>3/7</td>
<td>42.9</td>
<td>give, arizonae C</td>
</tr>
<tr>
<td>E</td>
<td>1/3</td>
<td>33.3</td>
<td>give</td>
</tr>
<tr>
<td>F</td>
<td>2/3</td>
<td>66.7</td>
<td>give, nottingham</td>
</tr>
<tr>
<td>G</td>
<td>5/6</td>
<td>83.3</td>
<td>give, arizonae C</td>
</tr>
<tr>
<td>TOTAL</td>
<td>17/31</td>
<td>x = 54.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>% Incidence = (# of isolations/# of attempts) X 100%.

<sup>b</sup>See footnote, Table 1.
**TABLE 3 - OAK CREEK SALMONELLA MPN AT SITE 3: APRIL, 1978 to APRIL, 1979**

<table>
<thead>
<tr>
<th>DATE</th>
<th>MPN/LITER</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 Apr, 1978</td>
<td>14</td>
</tr>
<tr>
<td>3 May, 1978</td>
<td>0.5</td>
</tr>
<tr>
<td>26 Jun, 1978</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>17 Jul, 1978</td>
<td>1.7</td>
</tr>
<tr>
<td>21 Aug, 1978</td>
<td>1.3</td>
</tr>
<tr>
<td>10 Sep, 1978</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>18 Nov, 1978</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>25 Mar, 1979</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>11 Apr, 1979</td>
<td>0.6</td>
</tr>
</tbody>
</table>
**TABLE 4 - OAK CREEK WATER CHARACTERISTICS MEASURED DURING SURVIVAL EXPERIMENTS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity (X $10^3$ (\mu\text{mhos}))</td>
<td>2.7 - 4.3</td>
</tr>
<tr>
<td>Dissolved Oxygen (ppm)</td>
<td>6.0 - 9.5</td>
</tr>
<tr>
<td>Calcium as Ca(^{2+}) (ppm)</td>
<td>21 - 24</td>
</tr>
<tr>
<td>Nitrate (ppm)</td>
<td>&lt;0.6 - .13</td>
</tr>
<tr>
<td>pH</td>
<td>6.8 - 7.2</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>0 - 23</td>
</tr>
</tbody>
</table>
LITERATURE CITED


APPENDIX A: Survival of *Salmonella* in Sheep Feces

*S. arizonae* was found in sheep feces during this study; the sheep herd in the study area had a carrier rate of 38.6%. Because of the large volume of feces excreted yearly by these sheep while pastured in the study area (slightly greater than $10^4$ kg of feces), it was desirable to determine the survival time of *S. arizonae* in these feces. Significant survival times could allow time for the organisms to leach from feces to the creek via surface runoff during times of heavy rain and runoff conditions.

An *S. arizonae* isolate from sheep feces was grown for 24 h in a 35°C shaking culture of brain heart infusion broth. Cells were then washed twice in Standard Methods phosphate buffer, and diluted to approximately $1.5 \times 10^5$ cells/ml in a one liter volume of Standard Methods phosphate buffer. 500 g of sheep feces were added to the cell suspension, which was then blended at high speed for one minute in a sterile Waring Blender. In order to maintain the natural feces flora and to prevent changes induced by heat, the feces were not sterilized. Use of the indigenous *S. arizonae* for measurement of *Salmonella* survival potential in feces was precluded by the low numbers of this organism in the feces. Therefore, a large inoculum of this isolate was used in order that statistically significant changes in the numbers of *salmonella* present could be detected by the most probable number (MPN) technique used here. The *Salmonella*-feces suspension was placed in a sterile foil covered beaker and placed near the Oregon State University
35th Street Sheep Barns, to approximate the environment of the sheep pasture near Oak Creek.

Salmonellae were enumerated by a multiple-tube MPN technique. Five replicates each of 10 ml, 1.0 ml, and 0.1 ml aliquots of slurry were placed into 10 ml Tet. Double-strength Tet was used for 10 ml samples. Tet was incubated at 41.5°C for 48 h, and processed as described in Materials and Methods. MPNs, including 95% confidence intervals, are graphed with temperature vs. time in Figure 1. Temperatures were obtained from N.O.A.A., U.S. Department of Commerce.

There was a strong inverse correlation between temperature and MPN (r = -0.86), indicating the organisms persisted better at lower temperatures. S. arizonae persisted very well, and viable salmonellae remained after 50 d, when the experiment was terminated. Despite this surprisingly long persistence, this S. arizonae serotype was only isolated once from Oak Creek. It would appear that suspended organisms in subsurface and surface runoff (such as the runoff ditches in the study area) provide greater potential for non-point pollution than organisms which may be leached from feces of domestic grazing animals during surface runoff.
FIGURE 1. Relationship between temperature, and survival time of *S. arizonae* in sheep feces slurry
APPENDIX B: Survival of Salmonella in Water Stored in Redwood Tanks.

The ability of the opportunistic pathogen Klebsiella pneumoniae to persist and even multiply in redwood tank water is documented (1). The purpose of this experiment was to determine the ability of the enteric pathogen Salmonella to persist under controlled conditions in redwood tank water. It has been observed that while salmonellae are capable of metabolizing some cyclitols that leach from redwood, this utilization is inhibited by other, antagonistic compounds that leach out as well (H. Talbot, Ph.D. Thesis, Oregon State Univ., 1979). It was reasoned that although antagonistic compounds may prevent salmonellae from multiplying, the organisms may persist.

Using the membrane diffusion chambers described in Materials and Methods, survival of S. give in 90 liter redwood tanks (National Tank and Pipe Co., Portland, OR) under various retention conditions was compared. As a control, one chamber was placed in a five liter container of Oak Creek water. Tank #1 water was constantly being replaced with chlorinated tap water on a 3 d retention time. That is, every three days the entire volume of the tank had been replaced by tap water slowly being metered in. The free chlorine residual was 0.1 ppm. Tank #3 was similarly treated on an 8 d retention time (<0.1 ppm chlorine), and Tank #2 was stagnant (no chlorine), as was the Oak Creek water control.

The survival of S. give under these various conditions was measured by plating five replicate plates of PCA for each chamber each time a sample was taken. The results (Figure 1) show that salmonellae died off most rapidly in Oak Creek water. They also did not persist well
in stagnant tank water or in tank water on a 3 d retention time. However, a small number of salmonellae persisted in tank water under an 8 d turnover time up to 26 d, when the experiment was terminated.

The laboratory temperature, high in relation to in situ Oak Creek temperature, accounts for the rapid dieoff of *S. give* in Oak Creek water, but does not fully explain why persistence in this environment was poorer than in any of the tanks. It is surmised that the chlorine level in the 3 d retention tank was sufficient to be antagonistic to *S. give*, and that the concentrations of inhibitory compounds in both the stagnant and 3 d retention tanks were sufficient to antagonize *S. give* survival. The presence of both chlorine and inhibitory compounds in the 3 d retention tank account for poorer persistence in this tank than in the stagnant tank, where no chlorine residual was present. Tank #3, on an 8 d turnover, had a lower chlorine residual than Tank #1. The chlorine level, in conjunction with a retention time sufficient to slightly reduce the levels of antagonistic compounds, may account for the greater persistence of *Salmonella* in Tank #3.

Even though salmonellae do not replicate in redwood tank water, the results of this experiment show that under some conditions salmonellae may persist for long periods of time in this environment. It appears that even when tank water is chlorinated, fecal contamination of redwood tank water could result in the danger of *Salmonella* ingestion.
FIGURE 1. Survival of *S. give* in membrane diffusion chambers suspended in redwood tanks and clean creek water.

Salmonella give S6

- Tank 1
- Tank 2
- Tank 3
- Oak Cr. water

Tank 1 - 3 d retention time
Tank 2 - Stagnant
Tank 3 - 8 d retention time
Oak Cr. Water - Stagnant