

## Antibiotic-Resistant Bacteria in Drinking Water†

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We analyzed drinking waters from seven communities for multiply antibiotic-resistant (MAR) bacteria (bacteria resistant to two or more antibiotics) and screened the MAR bacterial isolates obtained against five antibiotics by replica plating. Overall, 33.9% of 2,653 standard plate count bacteria from treated drinking waters were MAR. Two different raw water supplies for two communities carried MAR standard plate count bacteria at frequencies of 20.4 and 18.6%, whereas 36.7 and 67.8% of the standard plate count populations from sites within the respective distribution systems were MAR. Isolate identification revealed that MAR gram-positive cocci (*Staphylococcus*) and MAR gram-negative, nonfermentative rods (*Pseudomonas*, *Alcaligenes*, *Moraxella*-like group M, and *Acinetobacter*) were more common in drinking waters than in untreated source waters. Site-to-site variations in generic types and differences in the incidences of MAR organisms indicated that shedding of MAR bacteria living in pipelines may have contributed to the MAR populations in tap water. We conclude that the treatment of raw water and its subsequent distribution select for standard plate count bacteria exhibiting the MAR phenotype.

The occurrence of multiply antibiotic-resistant (MAR) bacteria in the environment is certainly a well-known phenomenon (8, 14, 28). Many investigators believe that these drug-resistant organisms have become more common recently due to the extensive use of antibiotics in medicine and agriculture throughout the world (15, 17, 24, 32). Concern about this situation has also become more common, since the antibacterial value of drugs is threatened seriously by the increased prevalence of resistant bacteria (15, 17). This concern is particularly relevant in light of the discovery that resistance characteristics can be transferred to nonresistant recipient cells via R-factor plasmid vectors (23).

Grabow et al. (15) emphasized the need to review water quality standards as they relate to the spread of antibiotic resistance genes in water-borne bacteria carrying transmissible R-factors. In environmental settings polluted by human or animal waste or both, high frequencies of MAR phenotypes exist in the coliform and fecal coliform populations (5, 9, 11, 27). These environments include surface waters receiving runoff from lands occupied by livestock (8, 11, 18), polluted estuaries (2, 28), and contaminated water supplies (19, 26, 30).

The study of fecal indicators has dominated studies of MAR bacteria in water because of the association of these indicators with disease-causing genera of importance to public health and

hygiene. However, it is not uncommon to find standard plate count (SPC) bacteria in drinking water at frequencies more than 10,000 times the frequency of coliforms (13). There is evidence that SPC bacteria in marine and freshwater environments can possess the same kinds of antibiotic resistance patterns as total and fecal coliform populations (2, 18, 28). Also, it is known that clinical isolates of the SPC population, *Acinetobacter*, *Flavobacterium*, *Moraxella*, and *Pseudomonas* can be MAR and may carry transmissible R-plasmids (1, 10, 15, 29). Typically, these same genera are encountered in the SPC population, and they often constitute the SPC population of municipal drinking water supplies (13, 21, 25).

To date, little work has been done to assess the prevalence of drug-resistant bacteria in treated drinking waters and their relationships to the MAR populations in the respective raw, untreated source waters. This study was undertaken to investigate this relationship, and attention was focused on the MAR bacteria within SPC populations.

### MATERIALS AND METHODS

**Municipal water supplies and source waters used for sampling.** The waters of six communities in Oregon were sampled. Community A receives its water from two mountain streams that originate in relatively virgin, unoccupied drainage systems. This water is chlorinated as its only treatment and is then distributed to a sprawling residential area. Community B obtains its water from a large use/reuse river which

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has a number of upstream cities and major industries located on its banks. This water is chlorinated, permanganate treated in the summer months, flocculated and coagulated with alum, treated with lime, fluoridated, filtered through charcoal and sand, chlorinated again, and distributed. Community C obtains its drinking water from the treatment facility in community B and from a creek draining a forested area. The creek water is treated when turbidity readings are more than 1 nephelometric turbidity unit in the winter months. This treatment includes chlorination, alum coagulation, and filtration. When turbidity is less than 1 nephelometric turbidity unit, the creek water is only chlorinated. Community D receives its raw water from a relatively unpolluted river. Treatment is by chlorination and alum flocculation. Community E is a rural residential area that depends on well water. Pumped water is stored in reservoirs and distributed to homes without any treatment or disinfection. Community F obtains its water from the same large use/reuse river as community B. The raw water is chlorinated and passed through a slow sand filtration system.

**Collection of samples.** Water samples were collected on 17 days from 11 February 1980 to 14 July 1980 according to standard methodology (4). Raw water and finished drinking water samples were collected in 4-liter sterile bottles containing 4 ml of 10% sodium thiosulfate to neutralize any free chlorine residual. Field measurements of free chlorine residuals were made by using a model CN-66 HACH DPD colorimetric analysis field kit. Samples were brought to our laboratory on ice and were analyzed within 6 h of collection.

**Enumeration of SPC bacteria.** Appropriate volumes of each water sample were filtered through GN-6 Gelman gridded filters (pore size, 0.45  $\mu\text{m}$ ). SPC bacterial densities were obtained from filters that were placed on mSPC agar, incubated for 48 h at 35°C, and examined with a microscope at  $\times 15$  (31).

**Antibiotic resistance testing.** Colonies to be screened for antibiotic resistance were picked from the filters used to enumerate SPC bacteria. To ensure a random sampling, picking was begun in the upper left square of each filter grid and continued across the squares from left to right, from row to row, until as many as 150 colonies were collected. Each replica master plate contained mSPC agar upon which 25 isolates had been inoculated. After 48 h of incubation at 35°C, a wooden block holding 25 nichrome wires (24 gauge) was used to replicate colonies from the master plate onto plates containing Mueller-Hinton agar (Difco Laboratories) supplemented with antibiotics. Each master plate was also replicated onto a Mueller-Hinton agar plate without antibiotics as a growth control. Only isolates that grew on the Mueller-Hinton agar control plates were used in enumerations of MAR bacteria. Mueller-Hinton agar was used since it is the standard medium for analysis of antibiograms (34). The five antibiotics used in the replica plating were purchased from Sigma Chemical Co., St. Louis, Mo.; these antibiotics were sulfanilamide (350  $\mu\text{g}/\text{ml}$ ), streptomycin sulfate (15  $\mu\text{g}/\text{ml}$ ), kanamycin sulfate (25  $\mu\text{g}/\text{ml}$ ), chloramphenicol (25  $\mu\text{g}/\text{ml}$ ), and tetracycline hydrochloride (12  $\mu\text{g}/\text{ml}$ ). These concentrations were the concentrations of the active antibiotics exclusive of

any associated anions. Antibiotic stock solutions and media were prepared and stored according to recommended clinical procedures (34). Concentrated antibiotic stock solutions were made at least once a month and were stored at -75°C. Mueller-Hinton agar plates containing antibiotics were stored at 4°C and were used within 7 days of preparation.

**Purification and storage of isolates.** Each MAR isolate to be identified was picked from a plate containing one of the antibiotics to which it was resistant and was inoculated into tryptic soy broth (Difco) containing 0.3% yeast extract (Difco). After 24 h of incubation at 35°C, a loopful of this culture was streaked onto tryptic soy agar (Difco) containing 0.3% yeast extract, and the plate was incubated 24 h at 35°C. A single colony from this plate was then used for identification.

**Identification.** Isolates were identified by the scheme of LeChevallier et al. (21). Isolates were placed into genera or groups on the basis of cell morphology, colonial morphology, Gram stain, catalase and oxidase reactions, motility, urease and indole tests, and glucose fermentation and oxidation. *Pseudomonas* and *Alcaligenes* were grouped together. The API 20E system was used to confirm coliform identifications. The coagulase and thermonuclease tests (35) were used to identify the *Staphylococcus* isolates to species.

## RESULTS

We screened a total of 2,653 SPC bacteria from 92 drinking water samples collected from six Oregon communities. Of these, 33.9% were MAR (i.e., resistant to two or more of the screening antibiotics). Within the group of MAR isolates, 61.1% were doubly resistant, 23.4% were triply resistant, 12.7% were quadruply resistant, and 2.9% were quintuply resistant to the antibiotics which were used.

In communities A and B untreated source water and distribution water samples were always collected on the same day in order to compare the numbers and kinds of MAR SPC bacteria. We found increased frequencies of MAR types in distribution water samples compared with the corresponding untreated source water samples. Community A receives its water from mountain creeks, and chlorination is the only treatment. Figure 1 shows the frequencies of various MAR phenotypes found in the raw and distribution water isolates. Of 535 isolates from 12 raw water samples, 20.4% were MAR, and 36.7% of the 839 isolates from 31 drinking water samples examined were MAR. This difference is statistically significant at the 5% level based on a *t* test.

Community B obtains its water from a large use/reuse river, and the water undergoes chlorination, fluoridation, alum flocculation, filtration, and secondary chlorination. As Fig. 2 shows, 18.6% of 301 isolates from five raw water samples were MAR, whereas 67.8% of 261 iso-

lates from nine distribution water samples were MAR. This difference is significant at the 5% level based on a *t* test.

Community E is a rural residential area that does not treat or disinfect the well water distributed to homes. SPC bacteria were often present at levels of 30 to 40 cells per ml. In 22 samples examined, only 14.5% of the 702 isolates screened were MAR, and 83.0% of the MAR population was doubly resistant.

Distribution water samples from communities C, D, and F were also analyzed. In community C, we found 79 MAR bacteria among 288 SPC isolates (27.4%) from 10 water samples. Six water samples from community D contained 101 MAR SPC bacteria among 150 SPC isolates (67.3%) tested. In community F, there were 111 MAR bacteria among 295 SPC isolates (37.6%) in 11

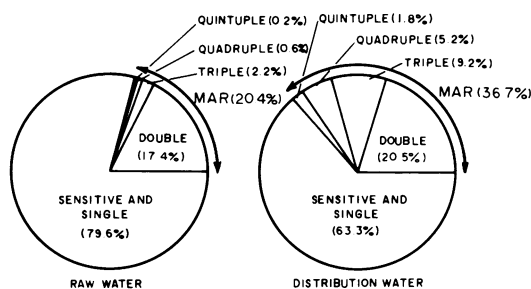


FIG. 1. Frequencies of sensitive, singly resistant, and MAR phenotypes among SPC bacteria from community A. Raw water was chlorinated as the only treatment before distribution. A total of 12 raw and 31 distribution water samples were filtered, yielding 535 and 839 isolates, respectively; these isolates were screened by replica plating.

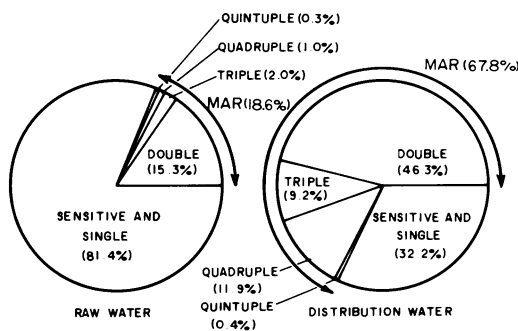


FIG. 2. Frequencies of sensitive, singly resistant, and MAR phenotypes among SPC bacteria from community B. Raw water was chlorinated, alum flocculated, charcoal and sand filtered, and secondarily chlorinated. Five raw and nine distribution water samples were filtered, yielding 301 and 261 isolates, respectively; these isolates were screened by replica plating.

samples tested. The incidences of MAR SPC bacteria in the corresponding raw water sources of these communities were not analyzed.

Representative SPC isolates exhibiting MAR phenotypes were identified. Table 1 shows the numbers of MAR isolates in different genera from the raw and distribution waters of communities A and B. Four major groups were identified according to Gram stain, fermentative metabolism, and cell morphology. The frequencies of MAR organisms in these four groups showed definite differences when the untreated and treated water isolates were compared. The most common MAR bacteria in the untreated waters were gram-negative, fermentative rods, and these represented 57.1% of the MAR isolates identified. Treatment and distribution of the water lowered the incidence of this microbial group to 3.8% and were accompanied by selection for MAR, gram-negative, nonfermentative rods and MAR gram-positive cocci. There was an increased incidence of the genera *Micrococcus* and *Staphylococcus*, the *Pseudomonas/Alcaligenes* group, and *Moraxella*-like organisms in the water after treatment.

One of the MAR isolates prevalent in the drinking water of community B was a *Pseudomonas/Alcaligenes* type that had yellow colonies and exhibited resistance to sulfanilamide and streptomycin sulfate. Because confirmation

TABLE 1. Identities and numbers of MAR SPC bacteria in raw and distribution waters of communities A and B

Identity	Raw water		Distribution water	
	No. of isolates	% of total	No. of isolates	% of total
Gram-negative, nonfermentative rods		38.1		55.7
<i>Acinetobacter</i>	7		18	
<i>Pseudomonas/Alcaligenes</i> group	11		79	
<i>Moraxella</i> -like group	17		56	
M				
<i>Flavobacterium</i>	0		1	
<i>Moraxella</i>	5		6	
Gram-negative, fermentative rods		57.1		3.8
<i>Aeromonas</i>	18		2	
<i>Citrobacter</i>	2		2	
<i>Enterobacter</i>	8		5	
<i>Hafnia</i>	29		1	
<i>Serratia</i>	3		1	
Gram-positive cocci		3.8		33.1
<i>Micrococcus</i>	1		32	
<i>Staphylococcus</i>	3		63	
Gram-positive rods		1.0		7.3
<i>Bacillus</i>	0		11	
<i>Arthrobacter</i>	0		2	
<i>Corynebacterium</i>	1		6	
Actinomycetes	0		2	

of the presence of this isolate was facilitated by its appearance and antibiogram, it could be traced as raw water was treated and distributed throughout the community. For example, on one occasion a raw water sample contained 7,930 SPC bacteria per ml. Of the 96 bacterial isolates obtained from this sample, 2 (2%) were yellow and resistant to sulfanilamide and streptomycin sulfate. On the same day, 79.6% of the 137 SPC isolates obtained from the clear well reservoir of the treatment facility (free residual chlorine, 0.7 mg/liter; SPC bacteria, 0.3 colony-forming units per ml) in community B had the yellow color and were resistant to sulfanilamide and streptomycin sulfate; isolates having this phenotype were also obtained on the same day from tap water samples in community B. The frequencies of MAR SPC bacteria independent of colonial color and specific MAR patterns were 27.1 and 86.1% for the raw water and clear well water, respectively.

Figure 3 shows the differences that we often observed between neighboring sites analyzed on the same day. In this case, water was taken from dwellings that were separated by about 400 meters (1,320 feet) on the same main line. Water sampled from site 1 contained an average of 22 MAR bacteria per 132 SPC bacteria (16.7%). At site 2, water contained 54 MAR per 103 SPC bacteria (52.4%). At site 1, 2.3% of the SPC

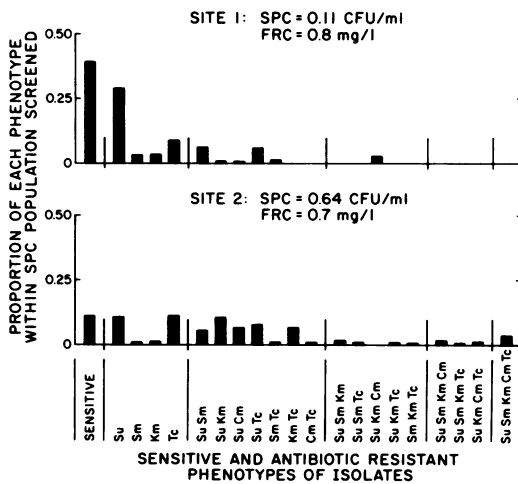


FIG. 3. Frequencies of sensitive and antibiotic-resistant phenotypes of bacteria from drinking water samples collected on the same day from two nearby sites in community A. CFU, Colony-forming units; FRC, free residual chlorine. The numbers of isolates screened from sites 1 and 2 were 150 and 133, respectively. Su, Sulfanilamide; Sm, streptomycin sulfate; Km, kanamycin sulfate; Tc, tetracycline hydrochloride; Cm, chloramphenicol.

bacteria were resistant to three antibiotics, and none was resistant to four or five drugs, whereas 12.6% of the SPC bacteria at site 2 were resistant to three, four, or five antibiotics.

We observed changes in the antibiotic resistance phenotypes and genera of MAR bacteria obtained from a single distribution outlet over a 3-month period. Figure 4 shows these variations in the frequencies of sensitive, singly resistant, and MAR bacteria. For example, on 17 March 1980 the Su<sup>r</sup> Sm<sup>r</sup> Tc<sup>r</sup> resistance phenotype comprised about 34% of all isolates examined, whereas this MAR category was not observed on 27 May 1980. This difference was statistically significant at the 5% level based on a z test comparing the two proportions. Also, on 27 May 34% of the isolates tested were quadruply resistant, whereas no quadruply resistant isolates were observed in the 17 March sample. The identities of the MAR bacteria in these samples also changed from month to month. On 19 February 13 of the 19 MAR isolates identified were *Staphylococcus*, and 6 were in the *Pseudomonas/Alcaligenes* group. All 12 of the MAR isolates identified on 17 March were *Staphylococcus*. On 27 May the 15 MAR bacteria identified included 12 gram-negative nonfermentative rods, 1 *Staphylococcus*, 1 *Micrococcus*, and 1 *Corynebacterium*. The raw source waters on 19

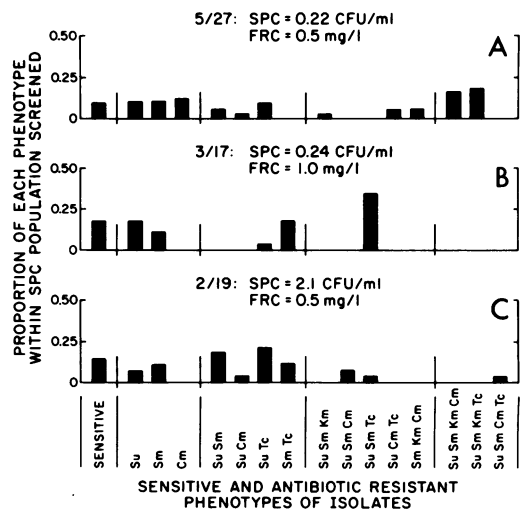


FIG. 4. Frequencies of sensitive and antibiotic-resistant phenotypes of bacteria from drinking water samples collected at a single site in community A on three different days. CFU, colony-forming units; FRC, free residual chlorine. The numbers of isolates screened on 27 May (A), 17 March (B), and 19 February (C) were 44, 29, and 28 respectively. Su, Sulfanilamide; Sm, streptomycin sulfate; Cm, chloramphenicol; Tc, tetracycline hydrochloride; Km, kanamycin sulfate.

February, 17 March, and 27 May carried bacterial populations which were 5.4, 23.0, and 6.1% MAR, respectively. On 19 February we obtained a *Staphylococcus* isolate from the raw water which was resistant to sulfanilamide and tetracycline hydrochloride, the same pattern observed for six *Staphylococcus* isolates taken from the drinking water on that day. Of 100 *Staphylococcus* drinking water isolates identified to species by the coagulase and thermoluciferase test, 4% were *Staphylococcus aureus*. Three of these were resistant to sulfanilamide and tetracycline hydrochloride, and the fourth was resistant to only sulfanilamide.

### DISCUSSION

This investigation documents the occurrence of MAR SPC bacteria in potable drinking waters. Overall, 33.9% of 2,653 SPC bacteria from finished drinking waters were MAR. This value may be a conservative estimate of the total MAR bacteria in drinking water since a larger number of screening antibiotics could have increased the efficiency of detection (18).

It was evident from comparisons of raw and treated water samples that treatment of raw water contributed to the enrichment of phenotypically MAR members in the SPC population. For example, one of the highest proportions of MAR SPC bacteria in all samples from all sites analyzed was observed in freshly treated water from community B, which was collected at the treatment facility. The water treatment process in community B is complex; the water is permanganate treated in the summer months, chlorinated, flocculated with an alum-lime mixture, fluoridated, filtered through charcoal and sand, chlorinated again, and then stored in a clear well at the treatment plant. This water contains 0.5 to 0.7 mg of free residual chlorine per liter. Replica plate screening of the SPC bacteria isolated from the clear well of this treatment facility indicated that 86.1% of these bacteria were MAR when 27.1% of the raw water SPC population was MAR. The pattern of double resistance to sulfanilamide and streptomycin sulfate expressed by members of the *Pseudomonas/Alcaligenes* group was especially common in the clear well water. Studies are now under way to clarify what aspects of water treatment at the facility in community B cause the enrichment of MAR SPC bacteria.

Overall, 67.8% of the SPC bacteria in the treated distribution water of community B were MAR. In community A, where water is only chlorinated, the average frequency of MAR SPC bacteria in the drinking water was 36.7%. The unchlorinated well water from community E

contained only 14.5% MAR SPC bacteria, a level which was similar to the level in the raw river water serving communities A and B. Thus, there was a correlation between the percentage of MAR bacteria and the extent of water treatment in the three communities studied in greatest detail. Whether this correlation also occurred in the cases of communities C, D, and F is not known since the raw water supplies of these cities were not studied. In community C, where river water is chlorinated, alum flocculated, and filtered through charcoal, the SPC population contained 27.4% MAR bacteria. Community D purifies river water by chlorination and alum flocculation, and 67.3% of the SPC isolates examined were MAR. An average of 37.6% of the SPC bacteria were MAR in water from community F, where river water is chlorinated and sand filtered.

The changes in the populations of MAR SPC bacteria when raw water supplies were treated were also reflected by changes in the identities of the predominant organisms constituting the MAR populations. Gram-negative, fermentative rods, such as *Aeromonas*, *Hafnia*, and *Enterobacter*, were killed very efficiently or removed or both during the treatment of raw water. A 15-fold decrease in the contribution of these organisms to the total MAR population (a decrease from 57.1 to 3.8%) occurred after treatment and distribution. There was also a corresponding increase in types such as the *Pseudomonas/Alcaligenes* group, *Acinetobacter*, *Moraxella*-like group M, *Staphylococcus*, and *Micrococcus*. Laboratory experiments are now under way to clarify the basis for this selection of MAR SPC bacteria.

Because the analysis of antibiograms is a sensitive tool for biotyping genera of SPC bacteria, it was used to demonstrate that the MAR bacteria were in a dynamic state of fluctuation within the distribution system. For example, an examination of the MAR bacteria isolated from two nearby sites revealed striking differences in types and frequencies. One site yielded water carrying MAR isolates at a 16.7% frequency, whereas 52.4% of the isolates were MAR at the second site. Population fluctuation was also observed at a single site sampled on a month-to-month basis. One of the factors that may have influenced this fluctuation was the shedding of cells from the resident population within a pipeline. Allen et al. (3) demonstrated colonization of distribution system pipes and used electron microscopy to visualize the cells within the microenvironment on the inner surfaces of water pipes. Concurrent isolation of specific genera with specific MAR phenotypes was observed in both raw and distribution water samples. This

suggests that population fluctuation may also be a result of different bacteria in the raw water that survive treatment. For example, in one of many similar cases, it was possible to recognize the *Staphylococcus epidermidis* isolates resistant to sulfanilamide and tetracycline hydrochloride obtained from a distribution site as belonging to the same biotype as the *S. epidermidis* strains isolated from the raw source water.

A calculation of the actual numbers of MAR SPC bacteria in finished drinking water indicated that a typical population of 100 SPC cells per 100 ml of drinking water contains 40 to 70 MAR bacteria. Even though this is considered a conservative estimate, such a low incidence may not be a general hazard to public health. However, there could be situations where the MAR SPC bacteria would act as opportunistic pathogens (33), and care should be exercised when exposing certain individuals to water containing MAR organisms. For example, *Pseudomonas aeruginosa* (12), *Flavobacterium* (16), and *Acinetobacter* (6), all of which are SPC bacteria, can cause disease. Someone who is receiving antibiotic or immunosuppressive drugs might become infected by MAR bacteria in the drinking water (33). The potential for infection in such a case is particularly relevant since the antibiotic concentrations used here to screen for resistant SPC bacteria are the levels accepted as constituting clinical resistance (22). The recent implication of *S. aureus* in toxic shock syndrome (7) generates immediate concern about the possibility that drinking water could be a source of this syndrome. The occurrence of MAR phenotypes among *S. aureus* reported here would be more health threatening if strains were also toxigenic, as was previously found for some drinking water isolates (20).

Above, we mentioned the widespread concern that the proliferation of antibiotic-resistant bacteria may hamper the efficacy of antibiotics in chemotherapy. We join others in this concern. In addition, we add a new dimension regarding the dissemination of MAR bacteria in the environment, namely, that disinfection, purification, and distribution of water may act as additional factors to augment the occurrence of drug-resistant bacteria. From the drinking water in a distribution system, MAR SPC bacteria pass via sewage to a waste treatment plant, where they may again be exposed to chlorination and other physical and chemical treatments. Then they are often returned to a river, perhaps the very source from which they originated when the water was processed originally into drinking water. Thus, an effective cycle for the selection of antibiotic-resistant bacteria is set up as the next

community downstream removes water from the river to repeat the process.

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