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CHAPTER **48**

Growth and Biocide Efficacy Studies Using the Iron-Oxidizing Bacterium *Gallionella*

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Procedures for evaluating biocides against the iron-oxidizing bacterium *Gallionella* have been developed. Biocide efficacies were evaluated on a consortium of *Gallionella* and two dominant pseudomonads. The optimum growth conditions for *Gallionella* were found to be an initial pH of 4.2 and a temperature of 15 ± 2 C. The amount of carbon dioxide added was not a critical variable except for the effect of CO_2 on pH. The concentration of heterotrophic pseudomonads also increased approximately 100-fold during a one- to two-week incubation in this minimal salts medium. Quantitation of biocide efficacy on *Gallionella* as well as the pseudomonads was done. The critical variable in biocide evaluations against *Gallionella* involves the time of biocide addition relative to the time of growth initiation. Of the two biocides evaluated, the formaldehyde condensate biocide was more effective against *Gallionella* when added early, whereas the quaternary ammonium biocide was equally effective at all times of addition.

INTRODUCTION

The iron-oxidizing bacterium *Gallionella* sp. is commonly found in iron-rich waters, particularly in cooler environments (Kucera and Wolfe 1957; Hanert 1981). The members of this genus are morphologically very complex with a small kidney-shaped cell attached to a long ribbon-like stalk (Kucera and Wolfe 1957; Hanert 1970). Recent analysis of stalk structure has determined the primary elements to be silicon, aluminum, calcium, and iron (Ridgway et al. 1981).

This organism appears to be one of the few obligate chemolithotrophs that is capable of deriving all of its energy requirements from the oxidation of ferrous iron and deriving its carbon from carbon dioxide (Hanert 1981; Buchanan and Gibbons 1974). The oxidation of ferrous iron to ferric iron results in the precipitation of ferric hydroxide; thus, the growth of *Gallionella* is typified by reddish mats of cells and ferric hydroxide encrusted stalks.

The growth of *Gallionella* in iron pipes is a problem in corrosion as well as clogging owing to the accumulation of cell-stalk masses (Miller and King 1975; Miller 1981; Ridgway et al. 1981). It would be important to determine the requirements for control of *Gallionella* growth in environments where corrosion and clogging are a significant problem (e.g., underground pipes used for oil field

injection waters or corrosion of metal surfaces that are difficult or impractical to replace).

This report presents the results of an evaluation of two biocides against *Gallionella* in a first attempt to develop appropriate methodologies for chemical control of this bacterium.

Materials and Methods

Isolation of Gallionella. The strain of *Gallionella* used for these studies was isolated from a dirt-floor basement in Macomb County, Michigan. This basement is approximately one mile from a 10-year-old landfill. The ground water height as well as direction of flow indicate that seepage from the landfill has contaminated the ground water supply and provided a rich and diverse environment for the growth of microorganisms. During the fall of 1983, the ground water level was near the soil surface, and the south wall and the floor of the basement were covered with a reddish layer covering black mud. Microscopic examination revealed that the reddish layer was composed predominantly of *Gallionella*. The ribbon stalks and ferric hydroxide particles were a significant portion of the total mass of the layer. The blackish mud layer contained high levels of metal sulfides, as determined by chemical analysis for sulfide. Samples of the upper reddish layer were used as the primary inoculum for the studies reported here.

Growth of Gallionella. The growth medium for culturing of *Gallionella* was a modification (Hanert 1981) of the medium of Kucera and Wolfe (1957). This modified medium contains 0.005% $K_2HPO_4 \cdot 3H_2O$ rather than the original 0.05% (Hanert 1981). This medium is a minimal salts medium with commercially available granular ferrous sulfide (Baker Chemical Co.). This product was found to be an excellent source of ferrous ions (hydrogen sulfide free) for the growth of *Gallionella*.

Ten ml of modified medium was added to 16-mm, screw-cap tubes and autoclaved. Sterile, filtered carbon dioxide was bubbled through the medium for 5 s to provide carbonate ions in solution. Approximately 0.3 g of ferrous sulfide was then added. The pH of the medium after 5 s of CO_2 addition was approximately 4.3. The tubes were incubated in a tap-water bath at 14–17 C. Flocculent *Gallionella* growth was observed on the sides of the tubes within 4 to 7 d (Fig. 1). The reddish material was stored at 3 C and inoculated into fresh medium at weekly intervals for 12 wk. Even after 12 wk of storage, viable *Gallionella* cells were easily detectable in the stored sample.

Transfer of Gallionella *cultures*. One- to two-week-old cultures of *Gallionella* (Fig. 1B) were used for preparation of test inoculum or for transfer. The tubes were rinsed five times with sterile deionized water and then resuspended in 10 ml of water by scraping the sides of the tubes with a pipet. The standard inoculum was 0.1 ml of such a suspension (Fig. 1B).

Experiments to trace the survival of heterotrophs (Fig. 3) involved a standard plate count of the supernatant of the *Gallionella* culture tube on plate count agar (DIFCO Laboratories) and a count of the attached microorganisms that were removed along with *Gallionella* by scraping the sides of the culture tubes.

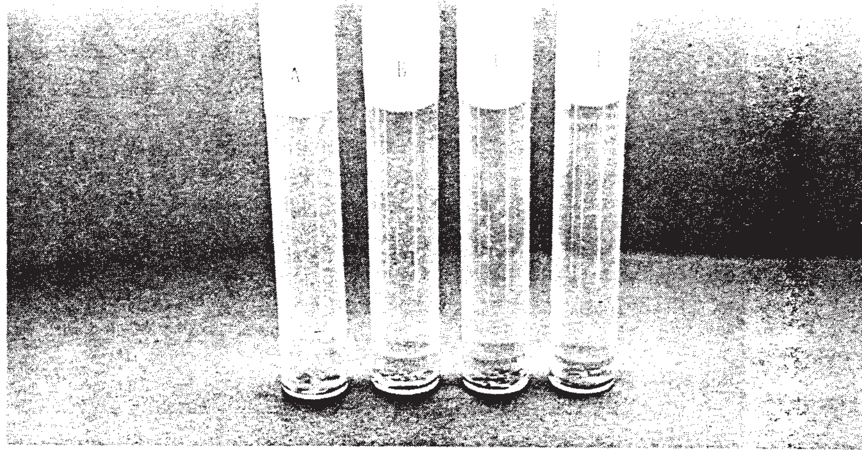

FIG. 1. Culture tubes of *Gallionella* after 10 d of incubation. Tube A was inoculated with 1 ml of a standard 7-d culture, and Tubes B thru D were 1:10 serial dilutions of this inoculum. The standard inoculum used for all testing is equivalent to Tube B. Macroscopic *Gallionella* colonies are visible on the sides of the culture tubes.

Results and Discussion

In order to evaluate the efficacy of biocides against *Gallionella*, the growth conditions were first optimized. The four parameters tested were pH, amount of CO_2 added, amount of FeS added, and temperature.

pH. Previous workers have reported that the growth of *Gallionella* should be optimal at a pH value between 5.5 and 7.6 (Hanert 1981; Kucera and Wolfe 1975; Nunley and Krieg 1968). The results of the experiment shown in Fig. 2 indicate that the strain of *Gallionella* used for these experiments has a broad pH range for growth from $\leq$pH 4.2 to pH 5.2. No growth was observed at pH 6.4, and growth was poor between a pH value of 5.2 and 6.4. The reason for the difference in these observations and previous literature reports is not apparent, but the strain of *Gallionella* used here not only prefers a low pH but also appears to be an acidophile. Bubbling CO_2 from 5 to 15 s into a standard tube results in a pH of 4.4 to 4.1.

Amount of CO_2 added. The amount of CO_2 was varied in several experiments from none added to 15 s of continuous bubbling per 10 ml of medium. Some small *Gallionella* colonies near the bottom of the culture tube were observed in the absence of CO_2 addition if the pH was decreased to 4.5 by the addition of HCl. Strong stimulation of *Gallionella* growth resulted from a small amount of CO_2 addition, but essentially no growth differences were observed between 3 s and 15 s of CO_2 bubbling. The critical variable appeared to be pH (Fig. 2) rather than the amount of CO_2 added. No growth occurred regardless of the amount of CO_2 added if the pH was increased to a value of 6.5 or higher by the addition of NaOH.

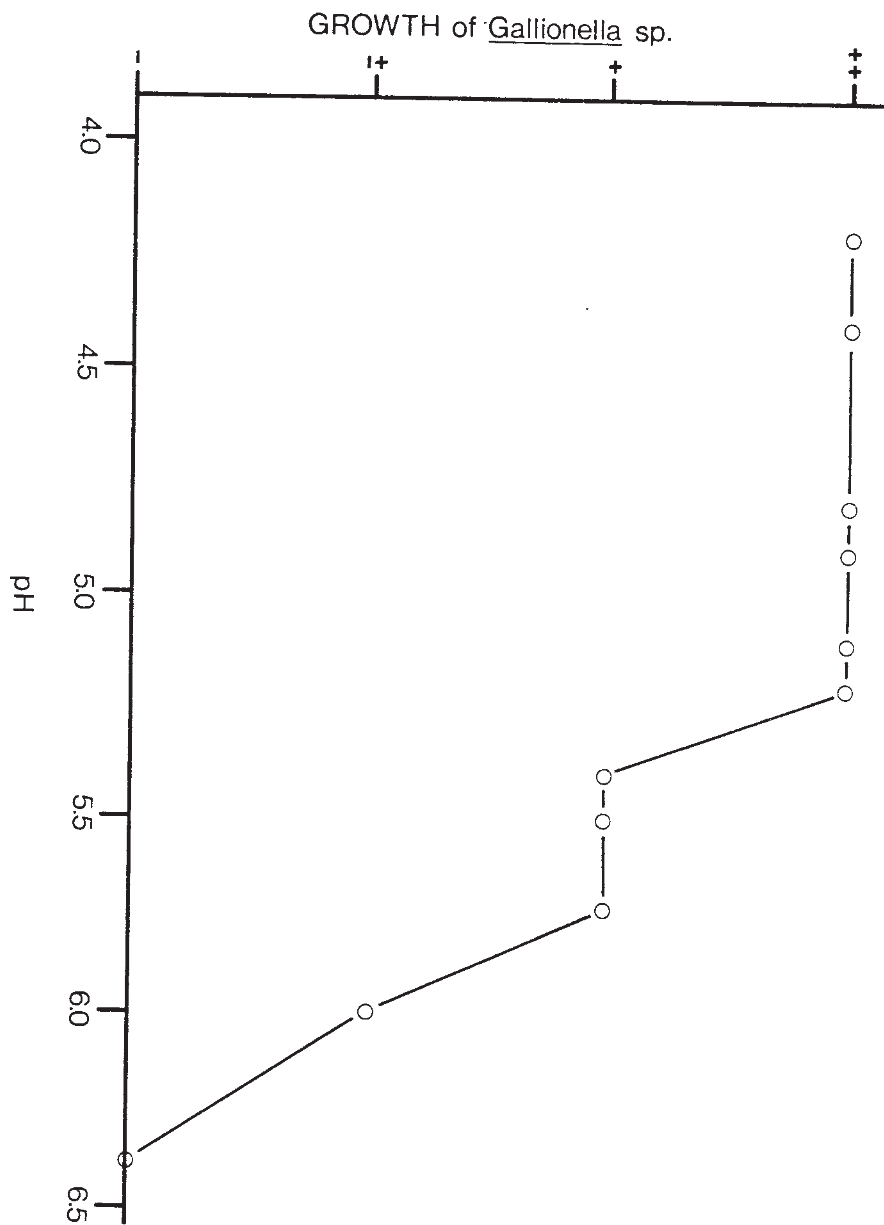

FIG. 2. The effect of pH on the growth of *Gallionella*. The results of three separate experiments are shown. The culture media contained varying amounts of carbon dioxide and the pH was adjusted with either NaOH or HCl. There was no effect on growth because of the amount of CO_2 added except as it affected pH.

KEY:

++ = heavy growth. Equivalent to Fig. 1A or 1B.
\+ = moderate growth. Less than or equal to Fig. 1C.
± = Poor growth. Equivalent to Fig. 1D.
− = No detectable colonies.

Amount of FeS added. No growth was observed in standard cultures in the absence of FeS. There was no observable difference in growth in cultures that contained between 0.05 g FeS and 0.5 g; therefore, approximately 0.3 g FeS was added to all standard test cultures.

Temperature. Four temperatures were used for testing the effect of temperature on *Gallionella* growth. The composite results of several tests are shown in Table 1. The strain of *Gallionella* used for these experiments is a psychrotroph similar to some of the strains previously used by others (Hanert 1981), whereas other strains have been reported to grow better at 25 C than at 20 C or lower (Kucera and Wolfe 1957). The incubation temperature used for all test cultures was 15 ± 2 C (tap water bath).

TABLE 1. *Effect of temperature on the growth of* Gallionella

Temperature	Incubation Conditions	7-Day Growth Observation[a]
3-5 C	Refrigerator	No growth
15-17 C	Tap water bath	200-300 Colonies per tube
22-24 C	Room temperature	100 Colonies per tube
29-30 C	Incubator	No growth

[a] No change occurs in the number of *Gallionella* colonies after 7 d.

Survival and growth of the heterotrophic members of the consortium. The microbial flora in the reddish layer of the dirt floor basement was partially characterized. At least 10 different morphological types of bacteria and 3 fungi were present. The black mud under the red layer contained at least 10^7 anaerobic sulfate reducers per cm^3. The environment from which *Gallionella* was isolated thus contained a rich heterotrophic microbial flora as well as anaerobic sulfate-reducing bacteria.

Hanert (1981) stated that he achieved pure cultures of *Gallionella* by repeated transfers of single washed colonies of *Gallionella*. For this method to be successful, some selection for growth of *Gallionella* versus attached heterotrophic bacteria would be needed; it is unlikely a single *Gallionella* cell would be obtained free of other bacteria unless there had been little or no growth of the initial heterotrophic contaminants during the week of incubation (Hanert 1981).

The results of an experiment to initially test this idea are shown in Fig. 3. Over the course of 10 wk, the concentration of heterotrophic co-contaminants present in the liquid phase of the tube (supernatant) as well as the heterotrophs that remain attached were determined. Initially (Fig. 3), there were less than 10^3 co-contaminants/ml introduced along with *Gallionella* in a diluted suspension of the primary inoculum (from the red layer described above). After the first 2 wk, there were 10^5 supernatant contaminants/ml and almost 10^6 attached co-contaminants/cm^2 of glass surface. After 2 wk, and at all subsequent time points, the resuspended attached heterotrophic population (about 10^6 cfu/ml) and approximately 10^4 *Gallionella* cfu/ml were diluted 1:100 during transfer. Even after repeated transfers and 100-fold dilutions, the supernatant and attached population grew to achieve the original population density (10^5 to 10^6 cells per ml or per cm^2).

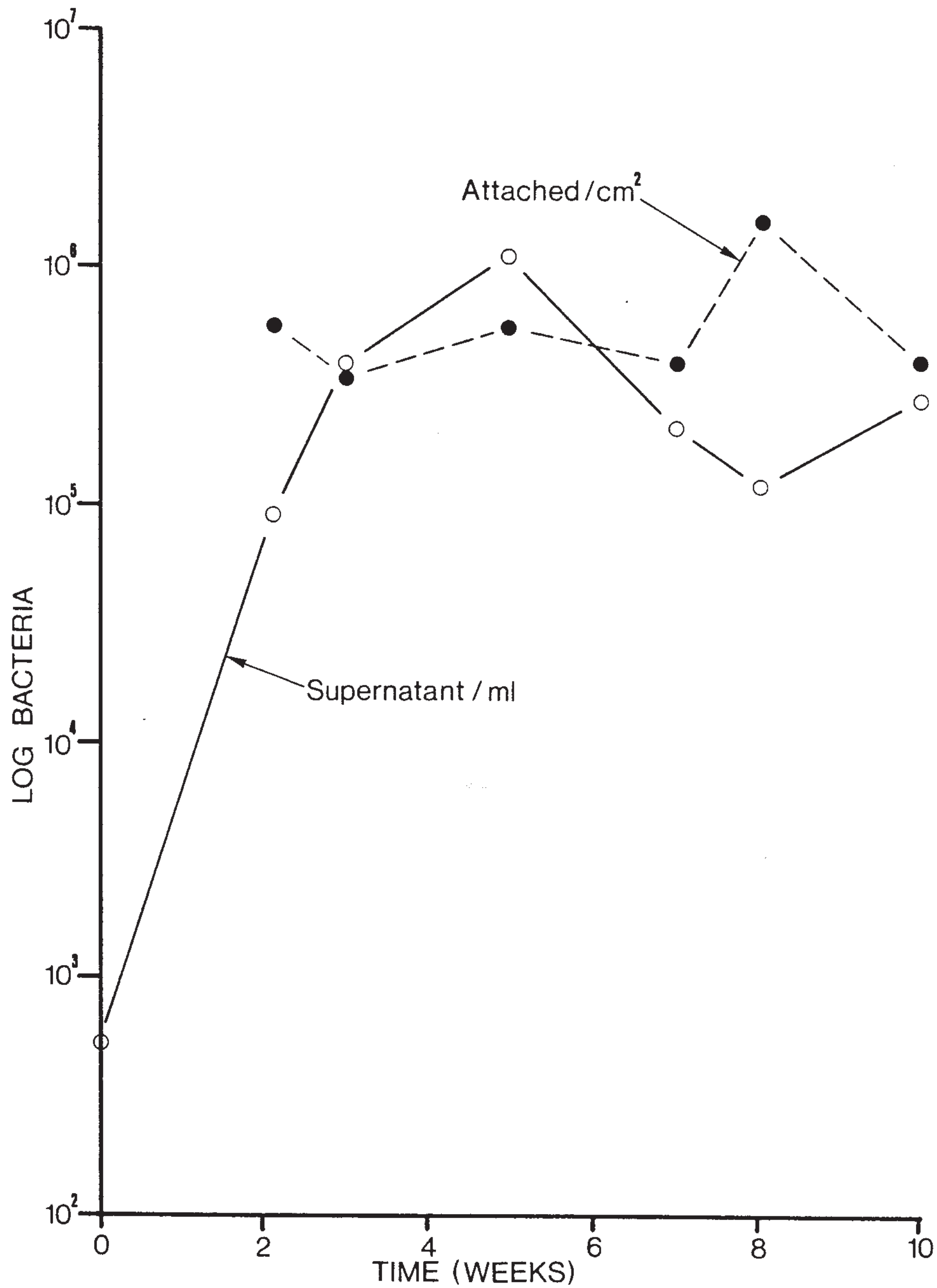

FIG. 3. Growth of heterotrophic co-contaminants during sequential transfer of *Gallionella* cultures. Supernatant counts/ml (○——○); attached counts/cm² (●——●).

Thus, this minimal medium containing only CO_2 as an added carbon source does support the growth of heterotrophs. The nature of this population changed during the repeated transfers shown in Fig. 3 until only two dominant bacteria

were present and no fungi were detectable. The same two organisms that were found in the supernatant were found attached to the glass surface. Biochemical tests of these two organisms have not been successful in determining a species identification. They are oxidase-positive glucose nonfermentors and thus are pseudomonas-like but not yet identified.

Effect of biocides on Gallionella. The culture methods described above were used to evaluate two biocides against *Gallionella*. A minimal inhibitory concentration (MIC) type experiment did not seem appropriate since there are at least three parameters of *Gallionella* growth and survival that are valid to test. These three parameters are as follows:

(1) *Biocide added at time zero*. This method is equivalent to a standard MIC experiment and tests the effect of the biocide on *Gallionella* prior to attachment. An apparently effective concentration of biocide may have prevented attachment rather than been biocidal to the cells. The effect of the biocide is evaluated after 14 d of incubation.

(2) *Biocide added after 3 d of incubation*. This method tests the effect of the biocide on *Gallionella* independent of attachment. Growth is visible after about 4 d of incubation; therefore, attachment and the initiation of growth have occurred after 3 d of incubation. The effect of the biocide is evaluated after 14 d of incubation.

(3) *Biocide added after 7 d of incubation*. This method tests the ability of the biocide to kill fully developed *Gallionella* colonies. In order to evaluate this effect, biocide is added after 7 d and the culture tubes are then incubated an additional 7 d. (There is no visible effect of biocides on fully developed colonies.) The tubes are then rinsed five times with sterile water to remove the biocide, and the surface colonies are resuspended and survival tested by transfer to fresh media.

The results of such an experiment using a formaldehyde condensate biocide are shown in Fig. 4. The effectiveness of the biocide decreases as attachment and growth of *Gallionella* proceeds. The concentration of biocide needed to kill fully developed *Gallionella* colonies is two times the concentration required to kill cells prior to attachment (Fig. 4). The effect of the biocide was also evaluated against the heterotrophs present in the culture tubes. There was no effect on their survival at a biocide concentration of 50 ppm or less regardless of the time of biocide addition, but 100 ppm (not shown in Fig. 4) was significantly inhibitory.

A second biocide evaluated was a quaternary ammonium biocide. Unlike the formaldehyde condensate biocide, this biocide was effective at 1 ppm regardless of the time of addition (data not shown). Lower concentrations of this biocide (ppb) may show a similar time dependence of addition as did the formaldehyde condensate biocide. The differences observed in the effectiveness of these two biocides, however, may also suggest that a difference in test methods may be appropriate.

This report of a test method for evaluating biocides against *Gallionella* is preliminary. Much remains to be known about this complex bacterium and how best to control its growth in inappropriate environments. For example, the growth of *Gallionella* on metal surfaces should be examined. These initial studies were done using a glass surface, but corrosion and clogging due to *Gallionella* are

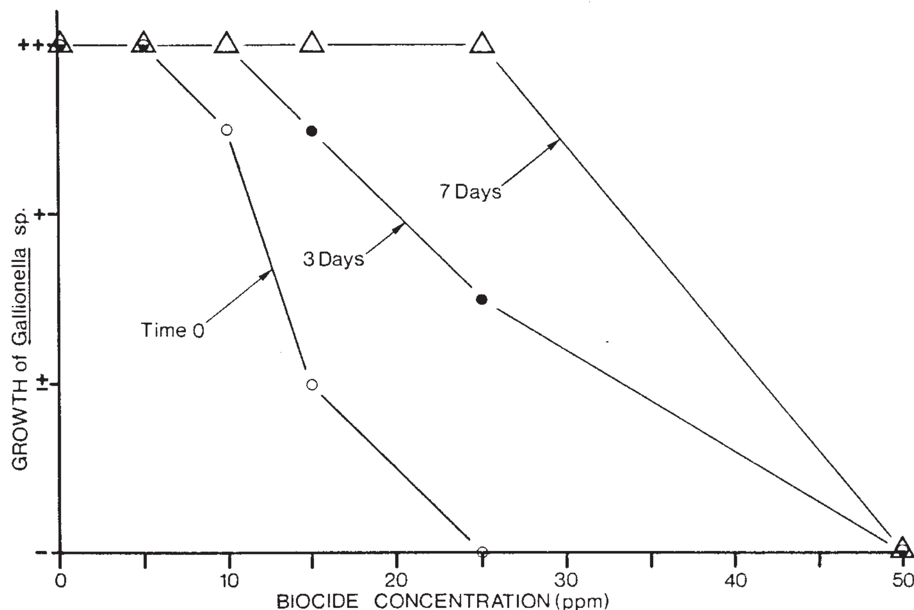

FIG. 4. Evaluation of a formaldehyde condensate biocide against *Gallionella*. The biocide was added to duplicate tubes at time zero or after 3 or 7 d of incubation. The time zero and 3-d experiments were evaluated for *Gallionella* growth after 14 d. The 7-d experiment involved 7 d of growth followed by 7 d of biocide treatment. This condition was then evaluated by determining the ability of the treated colonies to recolonize and grow in fresh media in the absence of biocide.

not likely to result from growth on glass. In addition, the parameters that are required to replace the requirement for ferrous ions provided by FeS with ferrous ions from an iron surface are not yet understood. Preliminary attempts to replace FeS with iron wire have been unsuccessful.

Gallionella is not uncommon in cold iron-rich waters, yet its growth in the laboratory is difficult to achieve. These bacteria are microaerophiles and grow best at low oxygen concentrations. The sometimes unavoidable variations in oxygen concentration were observed to result in growth as a narrow band of colonies near the bottom, middle, or top of the culture tube. This fastidious requirement for oxygen as well as the requirement for soluble ferrous iron suggests that growth of *Gallionella* in natural habitats may be enhanced by the presence of other microorganisms. For example, an iron-reducing *Pseudomonas* sp. (Obuekwe et al. 1981) might maintain a continuous supply of ferrous ions at low oxygen concentrations. Although there is no evidence for direct or indirect synergism between *Gallionella* and other microorganisms, the possibility exists that such interactions do occur and enhance the colonizing and survival capacity of *Gallionella*.

Thus, it may be appropriate for biocide test methods to include this possibility. If a biocide were effective against a synergistic heterotroph but not *Gallionella*, the laboratory test could incorrectly conclude that control of *Gallionella* during field use will be achieved. The biocides evaluated here using a mixed culture were

more effective against *Gallionella* than against the pseudomonad members of the consortium. This result is not necessarily a general rule nor should it be so interpreted until a greater understanding of the growth of *Gallionella* in natural habitats is achieved.

It has not been possible during these studies to achieve a pure culture of *Gallionella* (for example, by the use of formalin; Nunley and Krieg 1968). Should biocide evaluations be done only with pure cultures of *Gallionella*? Pure culture studies are invaluable for microbiology, but they should not be used when conclusions from such studies may be misleading.

Acknowledgments

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