DEVELOPMENT OF AN ENVIRONMENTALLY BENIGN MICROBIAL INHIBITOR TO CONTROL INTERNAL PIPELINE CORROSION

ELEVENTH QUARTER REPORT

(April-June, 2004)

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DOE Award No. DE-FC26-01NT41158

GTI Project No.:15317.1.01

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July 30, 2004

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ABSTRACT

Title:	Development of an Environmentally Benign Microbial Inhibitor to Control Internal Pipeline Corrosion
Funding Sources:	U.S. Department of Energy and Gas Research Institute
Contract No:	DE-FC26-01NT41158
GTI Project Nos:	15317.1.01/15054.4.05 (61138-01/30793-35)
Principal Investigators:	John J. Kilbane II, Ph.D. and William Bogan Ph. D.
Report Period:	April through June 2004
Objective:	The overall program objective is to develop and evaluate environmentally benign agents or products that are effective in the prevention, inhibition, and mitigation of microbially influenced corrosion (MIC) in the internal surfaces of metallic natural gas pipelines. The goal is to develop one or more environmentally benign (a.k.a. "green") products that can be applied to maintain the structure and dependability of the natural gas infrastructure.
Approach:	Previous testing indicated that the growth, and the metal corrosion caused by pure cultures of sulfate reducing bacteria were inhibited by hexane extracts of some pepper plants. This quarter tests were performed with mixed bacterial cultures obtained from natural gas pipelines.
Results:	Treatment with the pepper extracts affected the growth and metabolic activity of the microbial consortia. Specifically, the growth and metabolism of sulfate reducing bacteria was inhibited..
Conclusions:	The demonstration that pepper extracts can inhibit the growth and metabolism of sulfate reducing bacteria in mixed cultures is a significant observation validating a key hypothesis of the project. Future tests to determine the effects of pepper extracts on mature /established biofilms will be performed next.

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The main goal of this project is to develop an environmentally benign product that could prevent and/or control microbiologically influenced corrosion (MIC) in the interior of metal gas pipelines. The main activities for this quarter were the evaluation of hexane extracts of pepper plants to inhibit bacterial survival/growth and MIC, using mixed cultures of bacteria obtained from a natural gas pipeline.

Extracts from three varieties of *Capsicum sp*. (Chile de Arbol, Serrano, and Habañero peppers) were tested for their ability to prevent MIC in mixed cultures of microorganisms obtained from natural gas pipelines in the United States. The cultures contained natural mixtures of sulfate-reducing bacteria, acid-producing bacteria, denitrifying bacteria, iron-reducing bacteria, methanogens, and others. In a previous report, we observed that the hexane extracts of peppers were successful in inhibiting corrosion caused by pure cultures of sulfate-reducing bacteria, but unsuccessful in inhibiting corrosion by pure cultures of denitrifying bacteria. In this report, the testing of putative MIC-controlling biocides against naturally occurring mixed cultures is described.

Treatment with the pepper extracts affected the growth and metabolic activity of the microbial consortia. The pH of all samples decreased from initial values of 7.0 to 6.5 in untreated cultures and ≤6.2 in treated cultures during a six week period. This finding suggested the growth of acid-producing bacteria, and indeed, MPN analyses of the batch test revealed their presence. MPN analyses also detected the presence of SRB, but only in untreated cultures and samples treated with Habañero extracts. In general, protein production increased from initial values, but did not reach concentrations observed in the untreated control. In cultures treated with Chile de Arbol or Serrano extracts dentrification was the dominant process, followed by iron reduction; there was no significant sulfide production. By contrast, in cultures treated with Habañero extracts, dentrification was delayed by approximately 2 weeks and sulfide production occurred. Treatment with Chile de Arbol and Serrano extracts inhibited corrosion caused by the mixed cultures; treatment with Habañero extracts did not inhibit corrosion. These results suggest that the selective inhibition of SRB in mixed cultures decreases corrosion and that some pepper plant extracts, specifically hexane extracts from Chile de Arbol and Serrano peppers, appear to be

effective as environmentally benign alternatives to biocides for the control of corrosion due to MIC. Further testing to confirm and extend these observations is ongoing.

Additionally, a request for a no-cost time extension to continue this project until December 2004 was submitted to DOE after conferring with the DOE Project Manager.

BACKGROUND

The overall objective of this project is to develop, test, and apply environmentally benign agent(s) to control microbial-caused corrosion on the internal surfaces of metal (iron or steel) pipes used for natural gas production and transmission. The overall hypothesis being tested in this project is that agents exist in nature that inhibit some or all of the steps executed by microorganisms in the formation of biofilm and/or microbial processes resulting in metal corrosion. As biofilm formation is an absolute prerequisite for the initiation and production of microbiologically influenced corrosion (MIC), blocking biofilm formation or propagation will block or mitigate MIC. Similarly, if key metabolic processes of microorganisms that contribute to MIC can be inhibited then corrosion can be prevented/diminished.

The general approach is to evaluate natural products isolated from plants for their abilities to block the attachment, physiology, or reproduction of microbial agents that are responsible for MIC. Specifically, the natural products that are examined in this project are those compounds that can be extracted from the seeds and pods of pepper plants. These plants are members of the Genus *Capsicum.* The effective components or constituents of this product will then be tested for their environmental impact and effects, effective concentrations, modes of application, and stability against pure and mixed cultures of corrosion-associated microorganisms under simulated field conditions. A commercially viable agent that aids in MIC control and is environmentally friendly is the ultimate target, and this project will determine the feasibility of producing an environmentally benign MIC control agent derived from pepper plants. Additionally, other chemicals that may have the ability to selectively inhibit the metabolic processes of particular groups of microorganisms will also be evaluated to obtain information regarding the contribution of various microbial groups to corrosion.

Materials and Methods

Pepper Extract Stock Solutions. Three extracts were selected for batch tests. The extracts and the extraction procedure were described in previous reports. Extract 1 (obtained from Chile de arbol peppers) contained caffeic and coumaric acids as its primary identifiable constituents; extract 2 (Serrano peppers) contained several unknown organic acids; extract 3 (Habañero peppers) contained stearic, palmitic, and oleic acids. Stock solutions of 1%, 5%, and 10% of each extract were made in hexane, and inoculated into anaerobic growth media.

Media. Anaerobic growth medium was a modification of a previously described recipe (Daulton et al., 2002). Carbon sources/electron donors consisted of: lactate, glucose, succinate, glycerol, acetate, and citrate. Electron acceptors were: sodium nitrate, sodium thiosulfate, and iron (III). Yeast extract (0.01 %) was added to provide nitrogen and to stimulate initial growth. Finally, a reducing agent (1% thioglycollate and 1% ascorbate) was added to the final media at a ratio of 1:1000 ml.

Microbial Cultures. A mixed microbial culture was obtained from enrichment cultures of samples taken from a natural gas pipeline in Colorado (designated CO-WW081503). The cultures contained natural mixtures of sulfate-reducing bacteria (SRB), acid-producing bacteria, denitrifying bacteria, iron-reducing bacteria, methanogens, and others. Cultures were incubated anaerobically for one week in the aforementioned media prior to being used as inocula for experimental cultures.

Plate Counts and Most Probable Numbers Analyses. Microbial population size was estimated by standard plate counts (all organisms) and by Most Probable Numbers (MPN) analyses (SRB, acid-producing bacteria). For plate counts, 100 μl samples from each batch test were serially diluted and spread onto solid R2A agar plates (Difco Laboratories; Detroit, MI). Plates were incubated for 7 days in an anaerobic chamber after which plates having between 30 -300 colony forming units (cfu) were counted. The population size of the culture was estimated by multiplying the number of cfu by the dilution factor. For MPN estimates, a 1-ml sample of each batch test was added to 10 ml of modified Postgate's B medium or Acid-producing medium

(Dixie Testing & Products; Houston, TX); 1 ml of the resulting bacterial suspension was sequentially diluted by 10X increments to a final 10^{-5} or 10^{-12}-fold dilution. Each series was performed in triplicate. Anaerobic dilution tubes were scored for SRB by noting the presence of a black FeS precipitate after 14 days at 26°C. Anaerobic dilution tubes were scored for acid-producing bacteria by observing a color change in the medium from red to yellow (indicating a decrease in pH) after 14 days at 26ºC. MPN were determined using the program MOST PROBABLE NUMBER CALCULATOR© Version 4.04 (Klee, 1996).

Analytical Methods. Total protein was measured by the Lowry method (Lowry, 1951); sulfide was measured using the methylene blue method (Clesceri et al., 1998); iron (II) was measured by the ferrozine method (Stooky, 1970); and nitrite was measured using the method of Montgomery and Dymock (1961).

Metal Coupons and Weight Loss Measurements. Circular metal coupons were purchased from a commercial source (Metal Samples Co.; Munford, AL). Coupons were made of 1018 steel, had a diameter of 0.400 inches, and were ⅛ inch thick. The density of the coupons was 7.87 g/cm^3 and the surface area was 2.64 cm^2. Prior to experiments, the coupons were weighed. Upon the conclusion of experiments, coupons were removed from the batch tests and cleaned according to ASTM standard G1-90 (for metals composed of iron and steel). The coupons were subjected to a series of six solutions, either acidic or basic and at varying temperatures. One modification was made to the ASTM method: 5M sodium hydroxide was used at a temperature of 100°C for the last cleaning step. The coupons were then rinsed in deionized water, allowed to dry at room temperature overnight in a dessicator, and then weighed the next day. The corrosion rate of the metal coupons were calculated according to the following formula:

$$\text{Corrosion Rate} = (K \times W)/(A \times T \times D) \quad (1)$$

where:

K = 3,450,000 (constant used to determine corrosion rate in mils per year)
T = time of exposure (h)

W = weight loss (g)
D = density (g/cm^3)
A = surface area (cm^2)

Experiments. Sterile 125-ml serum bottles were filled with 99 ml of growth media and a metal coupon on the benchtop. The media was amended with 1 ml of 1, 5, or 10% of each pepper extract; the final concentrations of the extracts were therefore 0.01%, 0.05%, and 0.1%. The flasks were swirled to mix the extract and media. The media was inoculated with 1 ml of starter bacteria culture and the flasks were swirled again to distribute the organisms. Immediately thereafter samples were withdrawn to estimate the initial bacterial culture density and the initial concentrations of protein, sulfide, iron (II), and nitrite as previously described. The cultures were allowed to become anaerobic and samples were withdrawn aseptically at bi-weekly intervals to assay for culture density and metabolites. The experiments were concluded after 6 weeks and the coupons' weight loss was measured. Control cultures – inoculated but lacking pepper extracts – and sterile (non-inoculated) media controls were also performed. Each test was performed in triplicate.

RESULTS AND DISCUSSION

Figure 1 shows the corrosion rates for the Mixed Culture CO-WW081503 with 0.01, 0.05, and 0.10% (final concentration) of the 3 pepper extracts. The corrosion rates in cultures treated with Chile de Arbol or Serrano extracts were not significantly different than the sterile media control whereas the corrosion rates in cultures treated with Habañero extracts were higher. Unexpectedly, the corrosion rates in untreated cultures (bacteria-only controls, with no pepper extract) were significantly lower than the treated samples or in the sterile media. The reasons for this observation are not clear, as previous tests in our lab using pure laboratory strains resulted in relatively high corrosion rates. One possibility is that corrosion by naturally occurring microbial consortia is not as rapid as laboratory test cultures and was therefore not observed within the time frame of our experiments (6 weeks). Future experiments are planned with other pipeline consortia to determine if the result observed for CO-WW081503 is site-specific, an experimental artifact, or a general phenomenon among pipeline consortia. Results will be forthcoming.

Treatment with the pepper extracts affected the growth and metabolic activity of the CO-WW081503 microbial consortia. Standard plates counts from samples taken from the untreated cultures (bacteria controls) estimated the culture density to be approximately 1 x 10^4 cfu ml^{-1} at the onset of the incubation period. The culture density increased to 1.2 x 10^8 cfu ml^{-1} at 2 weeks in the absence of pepper extract. In contrast, cell densities were two to three orders of magnitude - approximately 1 x 10^5 cfu ml^{-1}, 1 x 10^6 cfu ml^{-1}, and 1 x 10^6 cfu ml^{-1}- in cultures treated with Chile de Arbol, Serrano, and Habañero extracts, respectively, after 2 weeks. There was no growth detected in sterile media controls.

The pH of all samples during the six week incubation. This finding suggested the growth of acid-producing bacteria, and indeed, MPN analyses of the batch test revealed their presence. Densities of acid producing bacteria reached a maximum of 1 x 10^8 cells ml^{-1} in untreated control cultures, but were approximately 10 -100X lower in treated samples (data not shown). MPN analyses also detected the presence of SRB, but only in significant densities in untreated cultures (max. 6.0 x 10^5 cells ml^{-1}) and samples treated with Habañero extracts (max. 1.1 x 10^5 cell ml^{-1}). The presence of SRB in Habañero-treated cultures may explain the higher corrosion rate in these cultures compared to batch tests with the other two pepper extracts (Figure 1). The absence of SRB in cultures containing Chile de Arbol or Serrano is consistent with results detailed in the last Progress report, which showed that SRB in pure culture are rapidly killed by the pepper

extracts. The failure of Habañero extract to kill SRB in the mixed culture is a departure from past results, and will be discussed further below.

Figure 2 shows representative results of biochemical assays that were performed on the CO-WW081503 cultures. Cultures were sampled for total protein content, as well as ferrous iron (the product of iron reduction), nitrite production (an intermediate product of denitrification), and sulfide (the product of sulfate reduction). Untreated samples (bacteria control; Figure 2 A) showed a constant increase in protein content over the course of the incubation, indicating that a metabolically active microbial population was present in the cultures. The reduction of iron (III) and nitrate was rapid and occurred within the first 2 weeks; after 2 weeks, the concentration of nitrite decreased suggesting that nitrite was used as an electron acceptor in anaerobic respiration. Nitrite was further reduced, thereafter. After 2 weeks, sulfate reduction commenced and was a significant anaerobic process, as can be seen by the production of sulfide in the cultures. There was no significant reduction of iron, nitrate, or sulfate in sterile media controls (Figure 2B); however, a constant background protein concentration was detected in the sterile media, possibly due to organic components in the media. Background protein concentration was subtracted from the experimental measurements in order to obtain the values reported in Figure 2.

Figures 2C, 2D, and 2E show the biochemical results from CO-WW081503 cultures treated with 0.1% Chile de Arbol extract, 0.1% Serrano extract, and 0.1% Habañero extract, respectively. In general, protein production increased from initial values, but did not reach concentrations observed in the untreated control. In cultures treated with Chile de Arbol or Serrano extracts, dentrification was the dominant process at 2 weeks, followed by iron reduction; there was no significant sulfide production (Figures 2C, 2D). By contrast, cultures treated with Habañero extracts displayed a different chemical profile than cultures treated with the other 2 pepper products: dentrification was delayed until approximately week 4 and sulfide production occurred after week 2 (Figure 2E). Sulfide concentration was similar to that observed in the untreated bacteria control (Figure 2A). The production of sulfide in Habañero-treated cultures may explain the higher corrosion rate in these cultures (Figure 1).

Sulfate reduction in Habañero cultures was an unexpected observation, as previous work in our lab showed that pure cultures of SRB were highly sensitive to all of the pepper extracts, including this one. It is possible that the diversity of SRB species present in nature includes some that are not as sensitive to hexane extracts of pepper plants as the pure cultures of SRB (*D.*

vulgaris and *D. desulfuricans*) previously tested. Alternatively, it may be that growth/activity of other microbial groups within the mixed consortia altered the conditions in the media or detoxified the pepper extract, which allowed SRB to flourish as the experiment progressed. In this regard, it should be noted that, as described in the last Progress Report, growth of (and corrosion by) the nitrate-reducing strain *Comomonas denitrificans* was highest in the presence if Habañero extract. Therefore, testing with pure cultures indicated that hexane extracts of peppers, including Habenero extracts, inhibited SRB but stimulated the growth and activity of denitrifying bacteria. The experiments reported here using mixed bacterial cultures indicate that extracts of Chile de Arbol and Serrano peppers are superior to extracts of Habenero peppers as regards corrosion protection. Further work will have to be conducted to verify and better explain this finding, and to determine if the mechanism by which pepper plant extracts inhibit corrosion is by the inhibition of sulfate reducers and/or stimulation of denitrifying bacteria.

CONCLUSIONS AND FUTURE EXPERIMENTS

Preliminary data suggest that pepper extracts may be able to selectively inhibit at least some species of sulfate reducing bacteria, which may in turn be responsible for a decreased corrosion rate. The stimulation of denitrifying bacteria by pepper plant extracts may also play a role in minimizing corrosion. Tests of the efficacy of the pepper extracts in controlling corrosion related to iron-reducing bacteria and tests of the pepper extracts in the presence of other biocides (i.e., ammonium cerium nitrate) are on-going. Future experiments will further characterize the ability of pepper extracts to inhibit pure and mixed cultures of corrosion-associated bacteria and determine the contribution of various microbial groups to metal corrosion.

In addition an experiment to isolate glutaraldehyde resistant microorganisms from natural gas pipeline samples is underway. Glutaraldehyde is the most widely used biocide in the gas industry and the isolation of glutaraldehyde-resistant cultures from natural gas pipeline samples will highlight the need to develop alternative biocides/microbial inhibitors. Moreover, knowledge of which bacterial species may be frequently encountered in natural gas pipelines as glutaraldehyde-resistant cultures may lead to improved methods to characterize MIC.

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Figure 1. Corrosion Rates of CO-WW081503 cultures treated with solutions of Pepper Extracts from 3 separate pepper plant species. The final concentrations of the extracts were 0.01, 0.05, and 0.1%. Control cultures (with bacteria but lacking pepper extracts) and sterile media controls (no bacteria, no extract) were also conducted.

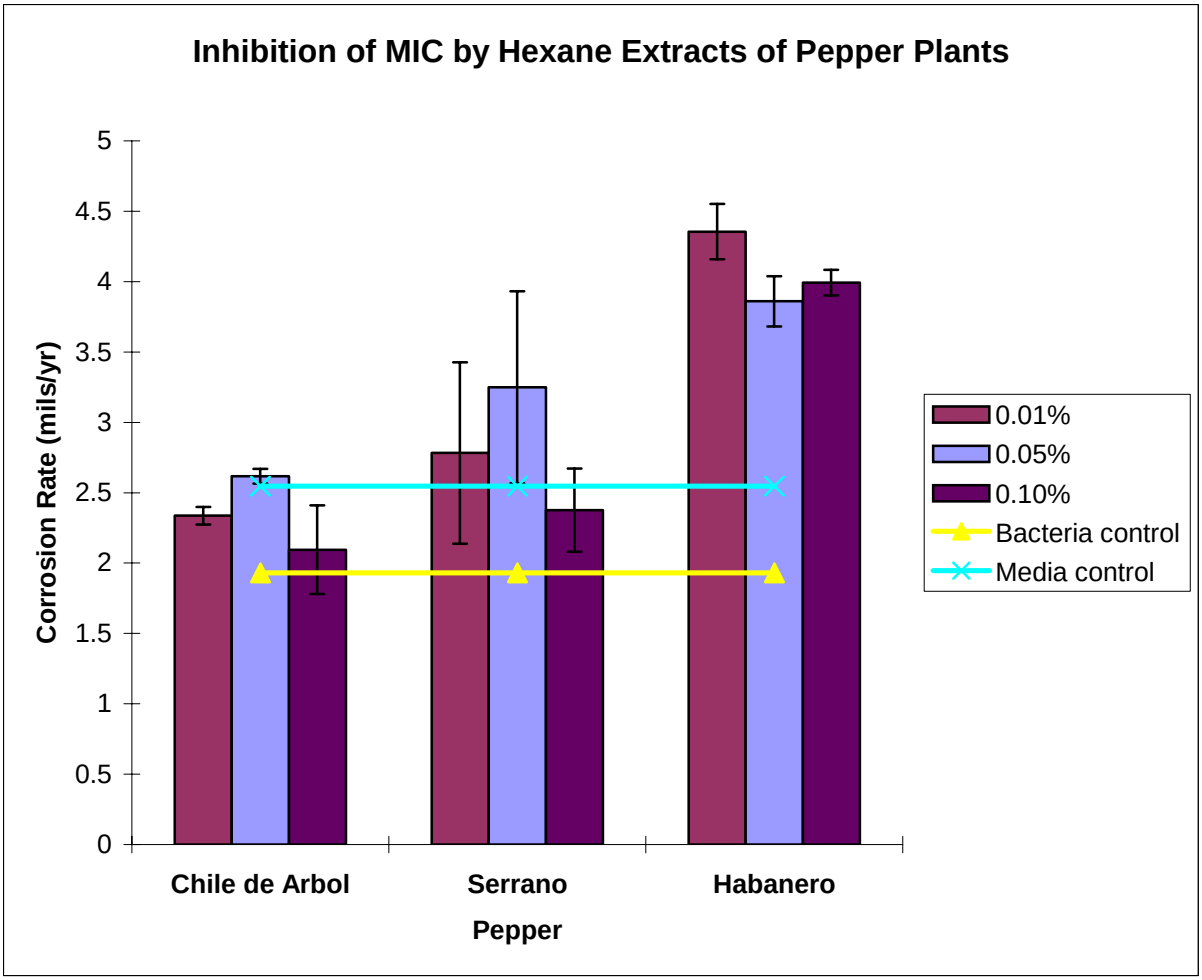

Figure 2. Chemical concentrations of CO-WW081503 cultures treated with solutions of Pepper Extracts from 3 separate pepper plant species. The final concentrations of the extracts were 0.01, 0.05, and 0.1%. Shown is a representative data set showing the results of tests with 0.1% extracts treatments. Control cultures (with bacteria but lacking pepper extracts) and sterile media controls are also shown.

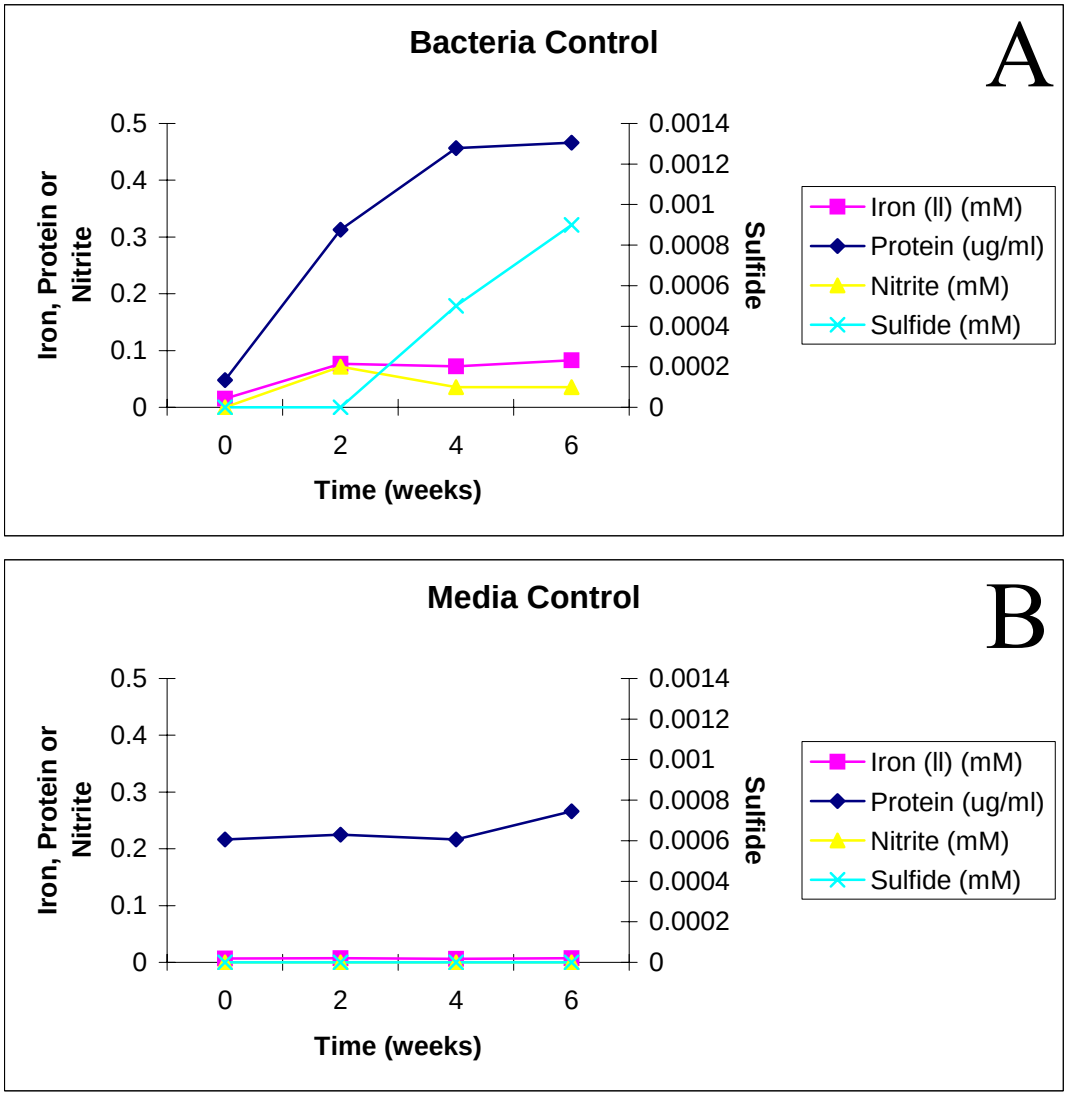

Figure 2 (cont.)