ENVIRONMENTAL BENIGN MITIGATION OF MICROBIOLOGICALLY INFLUENCED CORROSION (MIC)

FOURTH QUARTERLY REPORT

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Prepared by

J.R. Paterek

G. Husmillo

GAS TECHNOLOGY INSTITUTE

1700 South Mount Prospect Road

Des Plaines, Illinois 60018

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For

National Energy Technology Laboratory

626 Cochrans Mill Road

Pittsburgh, PA 15236-0940

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DOE Project Manager

Anthony Zammerilli

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DISCLAIMER

RESEARCH SUMMARY

Title: Environmental Benign Mitigation of Microbiologically Influenced Corrosion (MIC)

Funding Sources: U.S. Department of Energy and Gas Research Institute

Contract No: DE-FC26-01NT41158

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Principal Investigator: J. Robert Paterek, Ph.D.

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Objective: The overall program objective is to develop and evaluate environmental 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 one or more environmental benign, a.k.a. "green" products that can be applied to maintain the structure and dependability of the natural gas infrastructure.

Approach: The technical approach for this quarter were monitoring the development of *Desulfovibrio* species biofilm using the continuous flow cell system, evaluation of pepper compounds by microtiter plate assay for mitigating and inhibiting biofilm formation, and testing the effective concentrations to verify the extent of corrosion on metal coupons.

Results: Biofilm formation of *Desulfovibrio vulgaris* and *D. desulfuricans* was monitored and documented over a 7-day period. The use of a continuous flow cell system proved to be efficient and non-destructive in studying biofilm growth. Live/Dead *Bac*Light was an efficient stain to determine cell viability. The extracts showed 9-25% biofilm formation inhibition against the two organisms, and 18-19% activity in detaching the already formed biofilm. Preliminary data were obtained on the extent of corrosion of metal coupons when treated with pepper extracts as against the untreated ones. Confirmatory tests are underway. A presentation was prepared and give at the US DOE NETL meeting on gas and petroleum infrastructure. The presentation is include as an addition to this report.

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INTRODUCTION

The overall objective of this project is to develop, test, and apply environmentally benign agent(s) to control corrosion associated with internal surfaces of metal (iron or steel) pipes used in natural gas transmission. The overall hypothesis is that agents exist in nature that inhibit some or all of the steps executed by microorganisms in the formation of biofilm. As biofilm formation is an absolute prerequisite for the initiation and production of microbially influenced corrosion (MIC), blocking biofilm formation or propagation will block or mitigate MIC.

The general approach is to evaluate natural products isolated from plants, and possibly animals or microorganisms for their abilities to block the attachment, physiology, or reproduction of microbial agents that are responsible for microbiologically influenced corrosion. The first natural product to be tested is the oil that can be extracted from the seeds and pods of pepper plants. These plants are members of the Genus *Capsicum* and the first species evaluated was *Capsicum annuum*. The effective components or constituents (isolation and identification of these constituents in a previous and ongoing project funded by Gas Technology Institute) of this product will then be tested for its environmental impact and effects, effective concentrations, modes of application, and stability against isolated MIC microorganisms under simulated field conditions. A commercially viable agent that aids in MIC control and is environmentally friendly is the ultimate target with preliminary data to determine commercialization potential and cost-benefit analysis.

EXECUTIVE SUMMARY

The main goal of this project is to develop an environmentally benign compound that could prevent and/or control microbially influenced corrosion (MIC) in the interior of metal gas pipelines. Data presented is a summary of research activities from July 2002 to September 2002.

This quarter's activities were mainly focused on the all the research objectives with work progress especially on the third, fourth and fifth objectives, that is, determination of optimal environmental conditions for biofilm formation, evaluation of pepper components to inhibit biofilm formation and the identification of effective concentrations of the compounds tested. As discussed in previous reports, objective 1 is completed and objective 2 is progressing on schedule. Biofilm development was monitored using a continuous flow cell system. This proved to be an efficient way of monitoring biofilm development over time because of easy manipulation and fast documentation of results. Microtiter plate assay was used to verify the inhibition of *D. vulgaris* and *D. desulfuricans* biofilm formation by *Capsicum sp.* extracts and pure compounds. It was found that H1E, S1E and S1M extracts at 625 μg/mL showed 9 – 25% inhibition against the two organisms. Dissociation of the attached biofilm on microtiter plates was also evaluated using the same panel of test compounds. H1E at 625 μg/mL and S1E at 156 and 312 μg/mL detached 18.5% and 19 %, respectively, of the *D. vulgaris* biofilm. The same activity was noted for H1E at 156 μg/mL and S1M at 78 μg/mL, respectively, against *D. desulfuricans*. The extent of corrosion on metal coupons incubated with the extracts was monitored over time by weight loss determination. Preliminary data were obtained and more tests are underway.

EXPERIMENTAL

Objective 3 – Determine optimal environmental conditions for biofilm formation

Biofilm formation of the two strains of *Desulfovibrio* was studied using the continuous flow cell system (Stovall Life Science, Inc., Greensboro, NC) as shown in Figure 1. The sterile system consisted mainly of a 3-channel flow cell with individual channel dimensions of 1 mm D x 4 mm W x 40 mm L and the attached microscope coverslip #1 (0.13-0.16mm thick) that served as the substratum for biofilm growth. *Desulfovibrio vulgaris* and *D. desulfuricans* biofilms were grown in separate channels at 37^{o}C supplied with Modified Baar's Medium that was prepared under 97% N_2 and 3% H_2. Inocula used were from overnight cultures that were diluted to obtain a final cell concentration of 10^8 cfu/mL as measured at $OD_{600.}$ The medium was placed in a water bath and pumped into the system using the IsmaTec low flow peristaltic pump at a flow rate of 3mL/hr. The bubble trap near the influent side prevented the air bubbles from reaching the flow cell. After equilibrating the system with the culture media, the flow was stopped and the inoculum (0.5mL) was injected at the inlet of the flow cell. The flow cell was turned upside down to allow the cells to establish on the glass surface (Christensen, et al, 1999; Ramos, et al, 2001). After 1 hr, it was inverted to the right side up position and medium flow was re-started.

Biofilm development was monitored every 24 hrs for 7 days using light and epi-fluorescence microscopy (Carl Zeiss Axioskop 2 *plus*, Germany) and with Axiovision (Carl Zeiss, Inc.) as the software for image analysis.

To check for cell viability, two types of viable stains, 1mM 5-(and 6-) sulfofluorescein diacetate (SFDA) (Tsuji, et al, 1995) and Live/Dead *Bac*Light (both from Molecular Probes, Eugene, OR) were used. Injecting the dyes near the inlet of the flow cell stained the biofilms. Prior to injection, the cells were rinsed with distilled water and both ends of the flow cells were clamped.

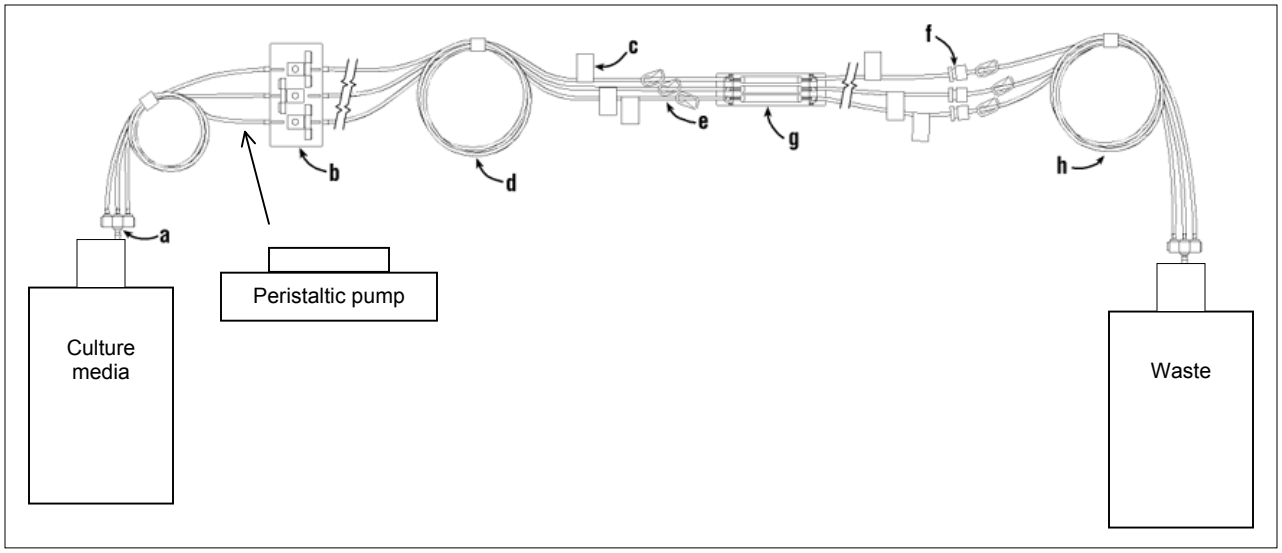


Figure 1. Diagram of the continuous flow cell system that was used to monitor biofilm formation

Legend:

- **a.** A three-outlet manifold disperses medium to a pump
- **b.** A triple cylinder bubble trap with air release cocks
- **c.** Write on flags
- **d.** PVC tubing connects the bubble trap to the flow cell
- **e.** Pinch clamps
- **f.** Luer connectors for collection of effluent
- **g.** Triple chamber flow cell with glass cover slip
- **h.** Tubing to waste container

Objective 4 – Evaluate pepper oil components to inhibit and mitigate biofilm formation and MIC

Microtiter plate assay was done to assess biofilm dissociation activity of *Desulfovibrio vulgaris* and *D. desulfuricans*. Biofilm formation was initiated by inoculating ATCC # 1249 Modified Baar's Medium was supplemented with 20% Oxyrase for Broth with *Desulfovibrio* cell suspension of 10^8 cfu/ml and incubating anaerobically at 30^{o}C. Media was replaced with fresh one after 96 hrs and plates were re-incubated for another 48-72h to ensure proper biofilm growth. Optical density was measured every 24h using the MRX II Plate (Dynex Technologies) plate reader. After the final incubation period, media was removed and 100 µl of the *Capsicum sp*. test compounds were added to appropriate wells then incubated for 1h. The wells were then rinsed with distilled water, air-dried and stained with 150 µl 1% crystal violet for 45 mins. They were washed with distilled water and de-stained by 200 µl 95% ethanol. Quantitative analysis of

the biofilm (Djordjevic, et al. 2002) present in the wells was done by transferring 100 μl of the de-stained biofilm into a new plate and measuring the optical density at 600 nm.

The same procedure was followed to assess inhibition of *Desulfovibrio* biofilm formation by the same *Capsicum sp*. compounds except that the test compounds were added from the beginning of inoculation and they were incubated with the two species to form biofilm up to 120h.

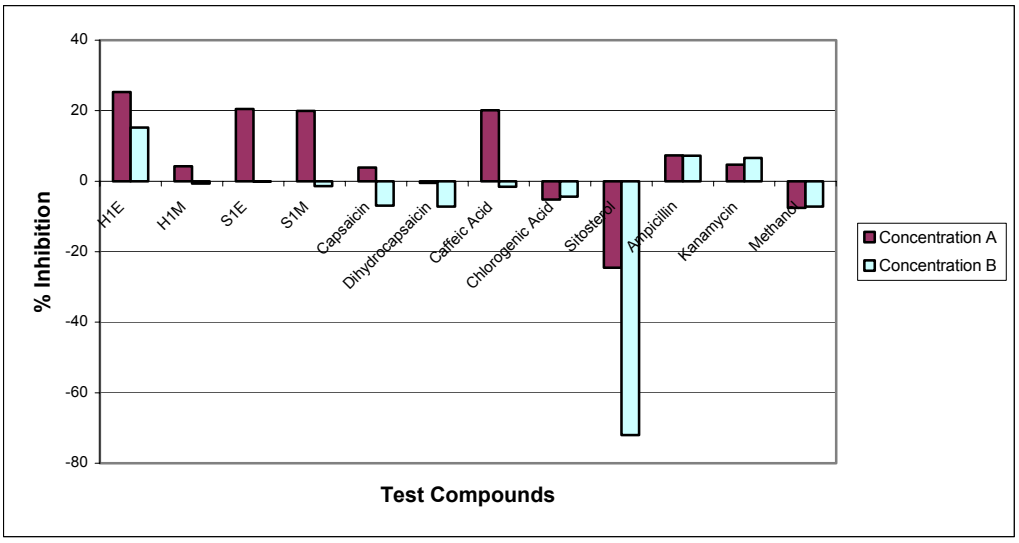
Objective 5 – Identify and test effective concentrations of pepper oil components

C1018 steel coupons (Metal Samples Co., Munford, AL) with a diameter of 7/8 inch and thickness of 1/8 inch were cleaned ultrasonically in ethanol for 1 min, degreased with acetone air-dried and stored under vacuum (Jayaraman, et al, 1997). They were weighed and placed inside rubber –stoppered flask and autoclaved using the gravity cycle (121^{o}C, 15 mins.). The flasks were incubated at 30^{o}C with 2% inoculum (v/v) from an overnight *D. vulgaris* culture in Modified Baar's Medium and the respective test compounds such as the pepper extracts (H1E at 312.5 μg/mL; H1M, S1E, S1M at 156 μg/mL), ampicillin (6.25 μg/mL), and methanol (0.625%). These final concentrations were based on the results of the minimum inhibitory concentration assay that was done previously. Duplicate coupons were harvested every 7 days while the media with the test compounds was replenished. The coupons were cleaned according to ASTM standards (ASTM, 1999) and weighed. The specific mass loss was determined in mg/cm^2 (for the total surface of the coupon, 3.87 cm^2) as an indicator of the extent of corrosion (Jayaraman, et al, 1999).

RESULTS AND DISCUSSION

The continuous flow cell system proved to be an appropriate environment to grow biofilms of *D. vulgaris* and *D. desulfuricans* since development could be studied in a nondestructive manner. Biofilm formation was monitored every 24h for 7 days and viability was verified using SFDA and Live/Dead *Bac*Light stains. SFDA was not absorbed by the cells, possibly due to its instability at environments with high moisture level, (Tsuji et al, 1995) and it also detached the formed biofilm. Live/Dead *Bac*Light, on the other hand, stained the cells with intact membranes as fluorescent green and those with damaged membranes as fluorescent red. This method will be adapted in the future biocide treatment on biofilms.

The effects of pepper extracts on SRB biofilm formation were determined using the 96-well plates as discussed previously. H1E, S1E and S1M extracts at 625 μg/mL demonstrated strong biofilm inhibiting activity with 20 – 25% inhibition against *D. vulgaris* (Figure 2a). The same concentration of capsaicin showed minimal effect and its lower concentration even promoted growth. However, for *D. desulfuricans* (Figure 2b), only S1E and S1M at 625 μg/mL manifested inhibition of 9 – 13%. Capsaicin and the other extracts enhanced biofilm formation of *D. desulfuricans* except for caffeic acid with about 50% at 1250 μg/mL and 32% at 625



μg/mL.

Figure 2a. Inhibition of *D. vulgaris* biofilm formation.

Data presented are averages of 3 replicates. Percent inhibition was computed based on the OD_{600nm} of the Untreated Cell Control. Concentration A = 625 μg/mL for extracts & capsaicin;

960 µg/mL for dihydrocapsaicin, 1250 µg/mL for caffeic and cholorogenic acid, 131.5 µg/mL for sitosterol, 200 µg/mL for ampicillin & kanamycin, 2.5% for MeOH. Concentration B= 50% dilution of concentration A.

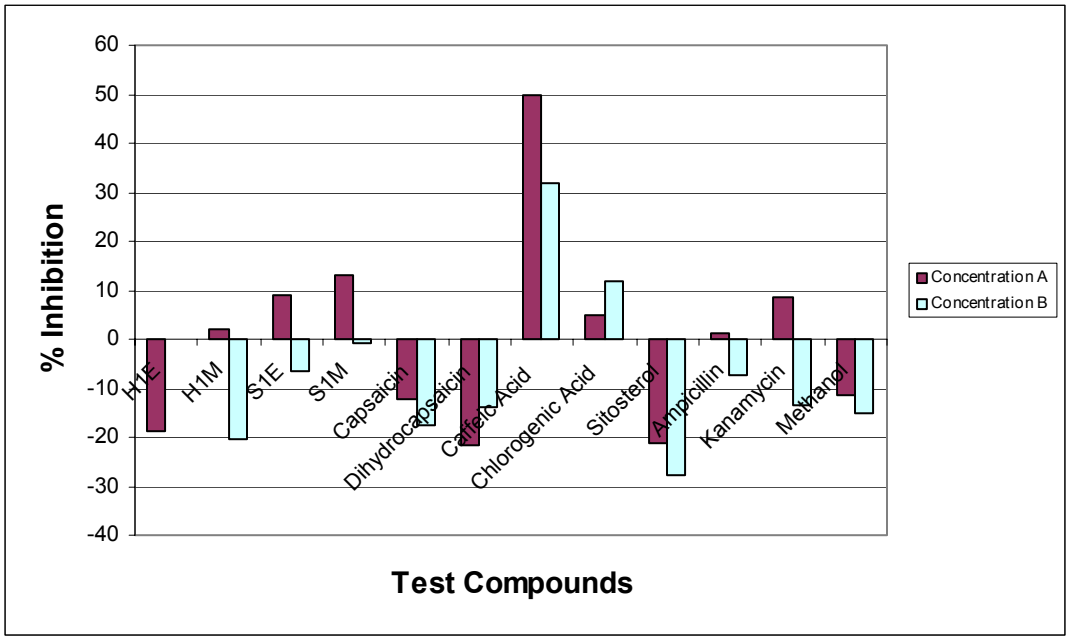


Figure 2b. Inhibition of *D. desulfuricans* biofilm formation.

Data presented are averages of 3 replicates. Percent inhibition was computed based on the OD_{600nm} of the Untreated Cell Control. Concentration A = 625 µg/mL for extracts & capsaicin; 960 µg/mL for dihydrocapsaicin, 1250 µg/mL for caffeic and cholorogenic acid, 131.5 µg/mL for sitosterol, 200 µg/mL for ampicillin & kanamycin, 2.5% for MeOH. Concentration B= 50% dilution of concentration A.

The extracts tested include H1E, H1M, S1E and S1M. The pure compounds include capsaicin, dihydrocapsaicin, caffeic acid, chlorogenic acid and sitosterol. Ampicillin and methanol were used as controls. Our data showed that among the ten compounds tested, C. *annuum* ether extract (S1E) at 156 and 312 µg/ml exhibited potency in detaching D. *vulgaris* biofilm from the substratum with 18.90% and 18.70%, respectively, difference from the untreated biofilm. C. *chinense* ether extract (H1E) at 625 µg/ml showed 18.49% difference from the untreated one (Figure 3a). The overall performance of all extracts was better than that of the pure compounds tested except for S1M at 156 µg/ml where biofilm formation was enhanced. Concentration range used for each compound varied depending on their Minimum Inhibitory Concentration (MIC) values from the antimicrobial screening that was previously reported. Per cent difference from the untreated group implies direct proportionality to detached biofilm.

Figure 3b shows the result of *D. desulfuricans* biofilm dissociation wherein the positive control ampicillin at 12.5 µg/ml and pure compound capsaicin at 312.5 µg/ml exhibited stronger activity than the crude extracts with 29.44% and 25.25% activity, respectively. S1M at 78 µg/ml and H1E at 156 µg/ml, however, demonstrated comparable activities detaching 18.90% and 18.47% formed biofilm, respectively.

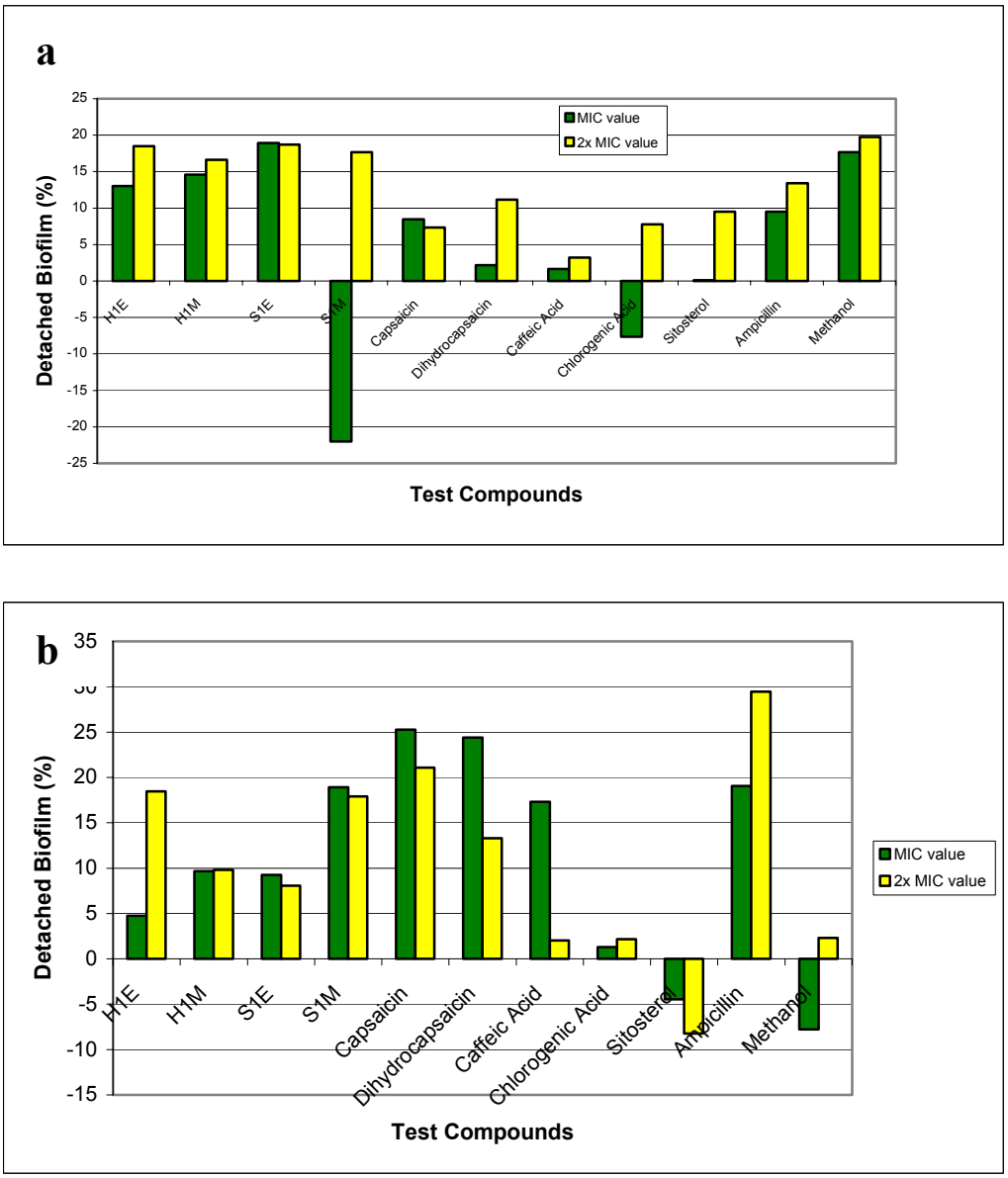


Figure 3. a) *D. vulgaris* biofilm dissociation. b) *D. desulfuricans* biofilm dissociation.

Concentrations used were based on the respective MIC values of each test compound. . Data presented are averages of 3 replicates.

Mass loss determination was done on metal coupons treated with the pepper extracts to check the extent of corrosion. Preliminary data were gathered and confirmatory tests are being done.

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