ENVIRONMENTALLY BENIGN MITIGATION OF MICROBIOLOGICALLY INFLUENCED CORROSION (MIC)

EIGHTH QUARTER REPORT

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DISCLAIMER

LIST OF ACRONYMS

Acronyms	Source Term
APB	Acid-Producing Bacteria
BAC	Biologically Active Compound
BCF	Billion Cubic Feet (of natural gas)
COS	Currently Off-the-Shelf (technology or products)
EETC	Emerging Energy Technology Campus
FERC	Federal Energy Regulatory Commission
GTI	Gas Technology Institute
GRI	Gas Research Institute
MCF	Million Cubic Feet (of natural gas)
MIC	Microbial Influenced Corrosion
MICC	Microbially Influenced Concrete Corrosion
MPN	Most Probable Number (statistical bacterial enumeration method)
PI	Principal Investigator
TCF	Trillion Cubic Feet (of natural gas)
SRB	Sulfate-Reducing Bacteria
HPLC	High Precision Liquid Chromatography
ESEM	Environmental Scanning Electron Microscopy

ABSTRACT

Title:	Environmentally Benign Mitigation of Microbiologically Influenced Corrosion (MIC)
Funding Sources:	U.S. Department of Energy and Gas Research Institute
Contract No:	DE-FC26-01NT41158
GTI Project Nos:	61138/30793-35
Principal Investigator:	J. Robert Paterek, Ph.D.
Report Period:	July 2003 through September 2003
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:	The technical approach for this quarter includes the application of the method of fractionation of the extracts by high performance liquid chromatography (HPLC); determination of antimicrobial activities of the new extracts and fractions using a growth inhibition assay, and evaluation of the extracts' ability to inhibit biofilm formation. We initiated the delivery system for these new biocides in the test cell and in mixtures of foam components and biocides/anti-biofilms.
Results:	A total of 51 fractions collected by HPLC from crude extracts that were obtained from three varieties of *Capsicum sp*. (Serrano, Habanero, Chile de Arbol) were subjected to growth inhibition tests against two SRB strains, D. *vulgaris* and D. *desulfuricans*. Five fractions showed growth inhibition against both strains while seven inhibited D. *desulfuricans* only. The crude extracts did not show growth inhibition on both strains but were proven to be potent in preventing the formation of biofilm. Growth inhibition tests of the same set of crude extracts against *Comamonas denitrificans* did not show positive results. The fractions will be subjected to biofilm inhibition and dissociation assay as well. The delivery system to be evaluated first was foam. The "foam pig" components of surfactants and water were tested with the biocide addition. The first chemical and physical parameters to be tested were pH and surfactants.

Conclusions:	Tests using the fractionated pepper extracts are progressing rapidly. Gas chromatographic analysis on a number of fractions is underway. Involvement of other microorganisms in the tests will be extended. The foam application method has been initiated and is being developed.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

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. The main activities for this quarter were focused on Objectives No. 4) to evaluate pepper oil components to inhibit and mitigate biofilm formation and MIC, and 5) identification and testing of biologically active compounds for biofilm inhibition or mitigation, 6) identification of commonly used chemical in pipelines, and 8) evaluation of potential deliver systems for biocides. In addition to the 12 new extracts from three varieties of *Capsicum sp*. (such as, Serrano, Habanero and Chile de Arbol) that were obtained by Soxhlet extraction using hexane, methylene chloride, aqueous acid, and aqueous alkali, an extract series using methanol and dimethyl sulfoxide (DMSO) solvent system was obtained. These extracts were subjected to growth inhibition tests and subsequently to fractionation by HPLC. The crude extracts did not show growth inhibition on both strains of *Desulfovibrio* but were proven to be potent in preventing their formation of biofilm. Growth inhibition tests of the same set of crude extracts against *Comamonas denitrificans* did not show positive results. Further, a total of 51 fractions were screened for growth inhibition against the two *Desulfovibrio* species. Five fractions showed growth inhibition against both strains while seven inhibited D. *desulfuricans* only. These fractions were then subjected to Gas Chromatography with Mass Spectrometry (GC/MS) in order to identify bioactive components. More fractions will have to be tested both for growth inhibition and biofilm formation while quantification of bioactive compounds is in progress. A number of pipeline chemical suppliers have been identified through business searches and discussion with National Association of Corrosion Engineers (NACE). Two to three chemicals (coatings, biocides, anti-corrosion chemicals and drying agents) of each class will be identified and the companies generating these chemicals will be notified. The delivery systems for the biocides (Objective 8) to the inside of pipelines activities were initiated. The first system chosen for testing uses stable or semi-stable foam containing the biocides. Based on Dr. Paterek experience in other GRI projects, a number of surfactants were identified for testing.

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 transmission. The overall hypothesis is that agents exist in nature that inhibits 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 MIC. 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.* The effective components or constituents 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 benefits.

Since the biocides have been identified, other chemicals that are used by the gas industry as biocides, anti-corrosion agents, drying agents, and coating are being evaluation for interactions. The suppliers have been identified, mainly through NACE, and they are being contacted to obtain samples (250 g to 500 g) of their chemicals.

The application of the pepper extract or its identified components using foams has initiated. This “foam pig” concept is accepted by the gas industry and some pipeline service companies are marketing this technology. GTI is looking a commercial foams and foams that are generated internally for a delivery system.

EXPERIMENTAL METHODS

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

Growth Inhibition (Antimicrobial) Screening

A total of 51 fractions from crude extracts that were obtained from three varieties of *Capsicum sp*. such as Chile de Arbol, Serrano, and Habanero by Soxhlet extraction with hexane, methylene chloride, aqueous acid and aqueous alkali, were collected by HPLC fractionation. They were subjected to antimicrobial screening against the two sulfate reducing bacteria, *D. vulgaris* and *D. desulfuricans*. The microdilution assay method (Eloff, 1998) was used to determine the growth inhibition of the pepper components. The test compounds were serially diluted 50% with sterile distilled water in 96-well microtiter plates. For the inoculum, an overnight bacterial culture was centrifuged at 4,000 rpm for five minutes, washed with a 50mM phosphate buffered saline, pH 6.8, and the optical density was adjusted at 600 nm to obtain a final cell concentration of 10^6cfu/mL per well. ATCC # 1249 Modified Baar's Medium was used as a source of cell nutrients. The multi-well plates were then placed inside a modular anaerobic chamber, purged with nitrogen gas, and incubated at 30^{o}C for *D. vulgaris* and 37^{o}C for *D. desulfuricans*. Optical density at 600 nm was measured using the MRX II (Dynex Technologies) plate reader from 0 up to 168 hours. Growth inhibition was determined as the test compound limiting turbidity to <0.05 absorbance at 600nm (Cai *et al.*, 2000). The initially acquired 12 crude extracts were also tested against the denitrifier *Comamonas denitrificans* using this procedure.

Objective 5 – Identify and Test Effective Concentrations of Pepper Oil Components

High-Precision Liquid Chromatography for Fractionation of Pepper Extracts

Pepper extracts were analyzed with High Performance Liquid Chromatography (HPLC) with a Photo Diode Array lamp (PDA) at 285nm.Extracts were received at a 10% dilution, this was done because the initial extract was too thick to inject, making it impossible to inject 250ul. Using the Supleco Supelcosil LC-18 column (25cm x 4.5mm x 5um) the analysis of the extracts was done with a flow rate of 1.0 ml/min and an initial solvent ratio of 80% A (0.01M Phosphoric Acid) and 20% B (HPLC grade Methanol). The flow rate remained the same but the solvent ratio

changed with time. The ratio changed gradually to equal 100% B by 57min of run time and then slowly went back to the original ratio by 66min. For each extract myristic, stearic, oleic, palmetic, caffeic, cinnamic, coumeric acids were qualitatively analyzed along with capsaicin and dihydrocapsaicin. These final two compounds will act as an internal standard and concentration marker for future testing. In addition, this was done to determine how to initiate the next step of the process, which was to fractionate the extracts.

The Waters Fraction Collector III was used for the fractionating. It is a programmable stand-alone fraction collection module. When running a sample many choices can be made from a simple process of operation such as time, drop, volume, or signal per collection vessel or a more advanced mode can be used such as peak mode, manual, and sampling mode. The time mode was observed to be sufficient.

The collector was set up to collect samples in 13 vials at certain time frames. These samples were collected in test tubes every 5 min. After all extracts were fractionated, they were evaporated to dryness using a mini-evaporator. Upon dryness, the extracts were given to the microbiologist for further analysis.

A sample each of Habanero with methylene chloride fractions were analyzed using gas chromatography with mass spectrometry (GC/MS) to determine what compounds are present.

Biofilm Formation

The crude extracts were tested against the two sulfate-reducing bacteria for their ability to inhibit biofilm formation. The methods used to look at the effects on biofilm formation and its measurements are discussed in detail by Djordjevic, *et al*. (2002), Fletcher (1999) and Horn and Jones (2002). The test compounds were incubated with bacterial cells at 10^8 cfu/mL (final concentration) and allowed to form biofilm up to 120 hrs. ATCC # 1249 Modified Baar's Medium was used as a source of cell nutrients. Biofilm assay was done by staining formed/residual biofilm with 1% crystal violet for 25 minutes, washing the wells with sterile distilled water and dissolving the cell-associated dye by adding 200 μL of dimethyl sulfoxide (DMSO) per well (O'Tode and Kolter, 1998). Quantification of the formed biofilm was done by optical density measurement at 600 nm using the MRX II (Dynex Technologies) plate reader and comparing the results with the untreated biofilm.

Continuous Flow Cell System

Biofilm formation of *D. vulgaris* was studied using the continuous flow cell system (Stovall Life Science, Inc., Greensboro, NC). 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. The TEST and CONTROL 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 each channel. The inoculum for the TEST channel was mixed pepper crude extract #5 while that of the CONTROL was suspended in the culture media only. 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 for the TEST channel was monitored every 4 hrs for 7 days using light microscopy (Carl Zeiss Axioskop 2 *plus*, Germany) equipped with time lapse program from Axiovision (Carl Zeiss, Inc.) as the software for image analysis. The Time Lapse software enables the camera to take photos at desired time intervals automatically for the whole duration of the experiment. For the CONTROL channel, photographs were taken manually at the beginning and the end of the run.

To check for cell viability, Live/Dead *Bac*Light (Molecular Probes, Eugene, OR) was used. The biofilms were stained by injecting the dyes near the inlet of the flow cell. Prior to injection, the cells were rinsed with distilled water and both ends of the flow cells were clamped. With epifluorescent microscopy, dead cells appear fluorescent red while live ones are fluorescent green.

Objective 6 – Identify and Evaluate commonly Used Chemicals by the Pipeline Industry for Interactions with Pepper Extracts and their Component Chemicals

A list of coatings, other biocides, corrosion inhibitors, dehydration compounds, and coating have been generated. A number of the manufacturers and distributors of these chemicals have been contacted and samples of these chemicals requested. Our pepper extracts and their components will be tested using the biofilm and biocidal testing described above within mixtures of these compounds. Results will be presented in the next quarterly report (41158R9).

Objective 8 – Evaluation of potential delivery systems for biofilm/MIC Mitigating Components as Foams, Coatings, or Injections

The first delivery system under evaluation is foams. The first testing will evaluate the effectiveness of the pepper extracts and their components with various surfactants. The pH is a critical issue that is being tested. Results will be contained with future quarterly reports.

RESULTS AND DISCUSSION

The Habanero peppers in methylene chloride extract was fractionated and then the extract evaporated to dryness. Once dried, all fractions were reconstituted with 500ul of the methylene chloride and injected in the HPLC using the previous conditions. Of the fractions, there were thirteen vials altogether but only vials 7-13 showed significant peak amounts. To determine what these peaks could be, the procedure was rerun using a GC/MS to determine some of the compounds (Table 1).

Table 1. Compounds detected in the Habanero fractions using GC/MS

Name	Formula	Molecular weight	Cas #
1,(2-aminophenyl)-3-methoxy-1-Propanone	$C_{10}H_{13}NO_2$	179	NA
N-butyl-Benzenesulfonamide	$C_{10}H_{15}NO_2S$	213	3622-84-2
N-oxide N-[(4-methylphenyl)methylene]-Benzenamine	$C_{14}H_{13}NO$	211	19865-55-5
Nonivamide	$C_{17}H_{27}NO_3$	293	2444-46-4
Natural Capsaicin	$C_{18}H_{27}NO_3$	305	404-86-4
Hexyl Vanillate	$C_{14}H_{20}O_4$	252	84375-71-3
bis(dihexylamide) Pentanediolic Acid	$C_{29}H_{58}N_2O_2$	466	NA

Like the HPLC, the GC/MS showed many peaks in vials 7-13 and none in 1-6. The above compounds were positively identified in those fractions. More analyses should be conducted using the Habanero pepper extract in hexane and then in DMSO/methanol as this would show which solvent would be more efficient in extracting the peppers.

The fractions collected by HPLC from crude extracts from three varieties of *Capsicum sp*. (such as, Serrano, Habanero and Chile de Arbol) that were obtained by Soxhlet extraction using hexane, methylene chloride, and 50% methanol with 50% dimethyl sulfoxide (DMSO) were used in antimicrobial screening against the two *Desulfovibrio* species. Table 2 lists the

summary of the results. Each crude extract yielded 13 fractions each except for extracts #7 –12 that were not fractionated due to inability of peaks to come out during initial HPLC runs. A total of 117 fractions were collected and samples to be tested were initially selected based on color and retention time. Out of 51 fractions tested, five showed growth inhibition against both strains while seven inhibited *D. desulfuricans* only. The rest of the fractions are now being prepared for testing.

The crude extracts did not show growth inhibition on both strains but were proven to be potent in preventing the formation of biofilm Figures 1 & 2. Growth inhibition tests of the same set of crude extracts against *Comamonas denitrificans* did not show positive results

Table 2. Growth inhibition by *Capsicum* sp. fractions against SRB

Extract No.	Fraction No.	Growth Inhibition	
		D. vulgaris	***D. desulfuricans***
1	**1**	**Yes**	**Yes**
1	**6**	**Yes**	**Yes**
2	**1**	**Yes**	**Yes**
2	**6**	**No**	**Yes**
3	**1**	**Yes**	**Yes**
3	**6**	**No**	**Yes**
3	**13**	**No**	**Yes**
4	**1**	**Yes**	**Yes**
4	**6**	**No**	**Yes**
5	**1**	**No**	**Yes**

5	**6**	**No**	**Yes**
AD	**1**	**No**	**Yes**

Extracts Legend:

1. Chile de Arbol with Hexane
2. Serrano with Hexane
3. Habanero with Hexane
4. Chile de Arbol with Methylene Chloride
5. Serrano with Methylene Chloride
6. Habanero with Methylene Chloride
7. Chile de Arbol with Aqueous Acid
8. Serrano with Aqueous Acid
9. Habanero with Aqueous Acid
10. Chile de Arbol with Aqueous Alkali
11. Serrano with Aqueous Alkali
12. Habanero with Aqueous Alkali

AD – Chile de Arbol with DMSO / Methanol
HD – Habanero with DMSO / Methanol
SD – Serrano with DMSO / Methanol

Fractions Legend:

1. Caffeic Acid, Coumaric Acid
2. *
3. Stearic Acid, Palmitic Acid, Oleic Acid
4. *
5. *
6. Cinnamic Acid
7. *
8. *
9. Capsaicin, Dihydrocapsaicin
10. Myristic Acid
11. *

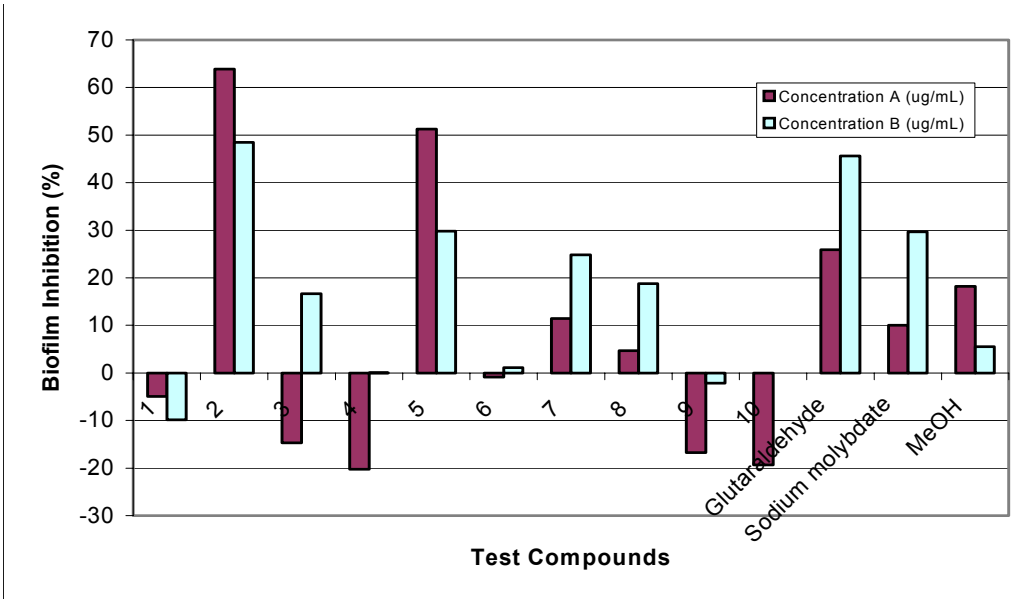

Figure 1. Inhibition of D. vulgaris Biofilm Formation

Concentration A = 500 μg/mL for extracts # 1-6, 1250 μg/mL for #7-9, 2500 μg/mL for #10 & glutaraldehyde, 5000 μg/mL for sodium molybdate, and 5% for methanol. Concentration B = 250 μg/mL for extracts # 1-6, 625 μg/mL for #7-9, 1250 μg/mL for #10 & glutaraldehyde, 2500 μg/mL for sodium molybdate, and 2.5% for methanol.

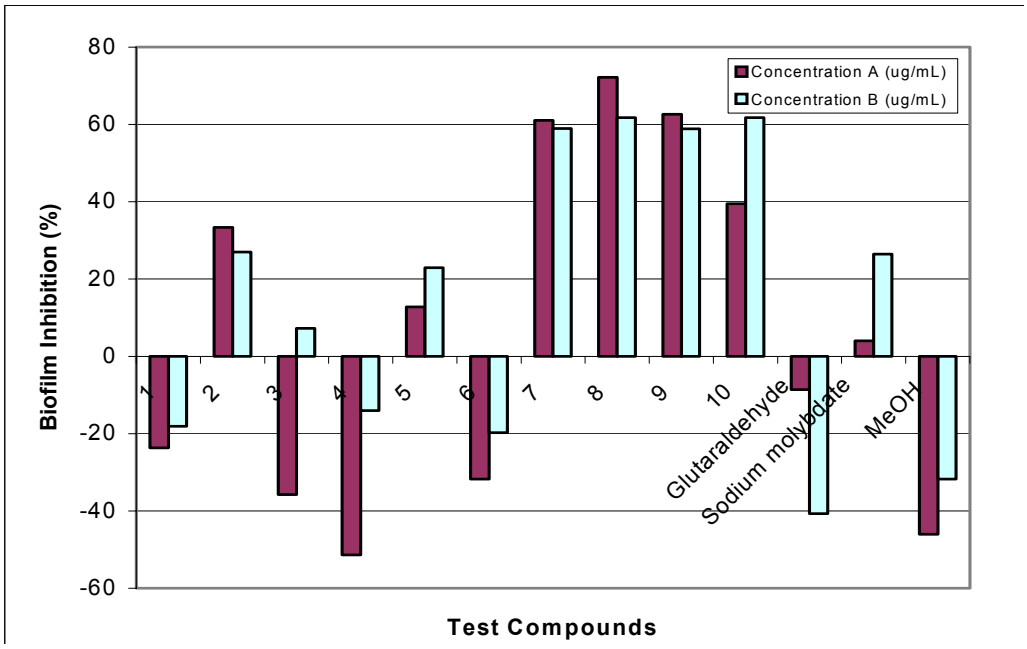

Figure 2. Inhibition of *D. desulfuricans* Biofilm Formation

Concentration A = 500 μg/mL for extracts #1-6, 1250 μg/mL for #7-9, 2500 μg/mL for #10 & glutaraldehyde, 5000 μg/mL for sodium molybdate, and 5% for methanol. Concentration B = 250 μg/mL for extracts # 1-6, 625 μg/mL for #7-9, 1250 μg/mL for #10 & glutaraldehyde, 2500 μg/mL for sodium molybdate, and 2.5% for methanol.

Based on the above results of biofilm inhibition against both microorganisms, the continuous flow cell system experiment was conducted. The system utilized extract #5 as the test compound and D. *vulgaris* as the test organism. Time Lapse Module of the Carl Zeiss' Axiovision 3.0 was used to document biofilm formation in the TEST channel wherein the extract was mixed with the microorganism to begin with. Biofilm growth was monitored and automatically documented every 4 hours for 168 hours (7 days) with the culture media continuously flowing through the system at a steady slow rate of 3.19 mL/hour. Since the software cannot be interrupted once started, the CONTROL channel that is simultaneously running but without extract has to be documented manually at the beginning (0 hour) and at the end (168 hours) of the system run. It is interesting to find out that with the presence of pepper extract, the cells appeared to be pleomorphic. Neither cellular reproduction nor change in morphology was observed until 72 hours when cell division started occurring. The cells then started to recover morphologically and multiply to form a monolayer at 120 hours. There was no significant growth increase from this point up to 168 hours when the experiment was terminated. In contrast, the cells in the CONTROL channel started forming clumps at the beginning. After 168 hours, they did not only form a monolayer, but layers of cells on top of each other. Figure 3

shows the cells in the CONTROL and TEST channels at a) 0 hour, and b) 168 hours. The cells in both channels were stained with BacLight Live/Dead probe to check viability with epifluorescent microscopy. Figure 4 shows the cells after staining wherein live cells fluoresce green and dead cells fluoresce red.

Control Test

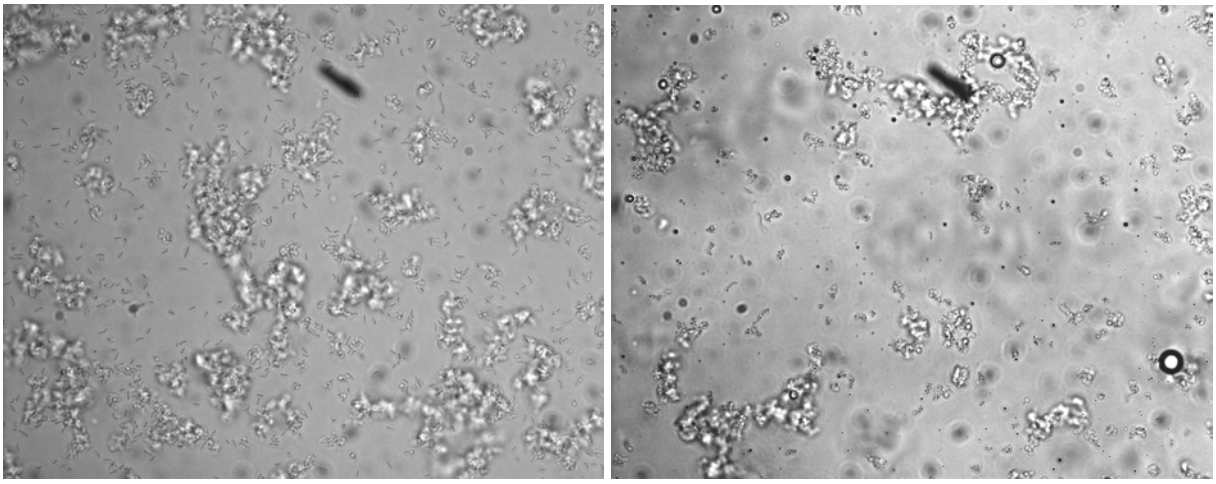

Figure 3a. Cells in the control and test channels at 0 hour. Viewed under oil immersion objective (Total magnification of 1000x)

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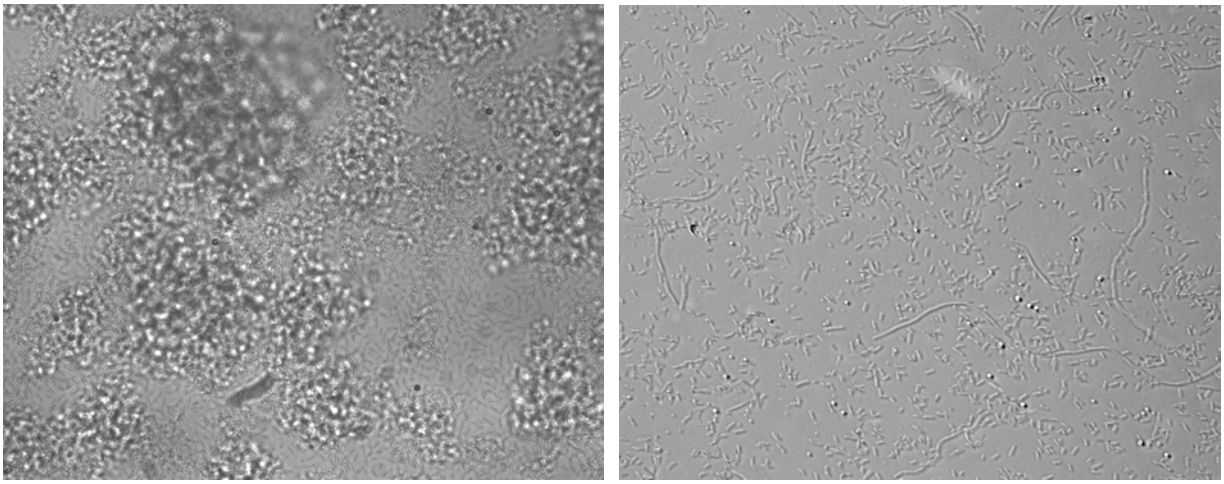

Figure 3b. Cells in the Control and Test channels after 168 hours. Viewed under oil immersion objective (Total magnification of 1000x)

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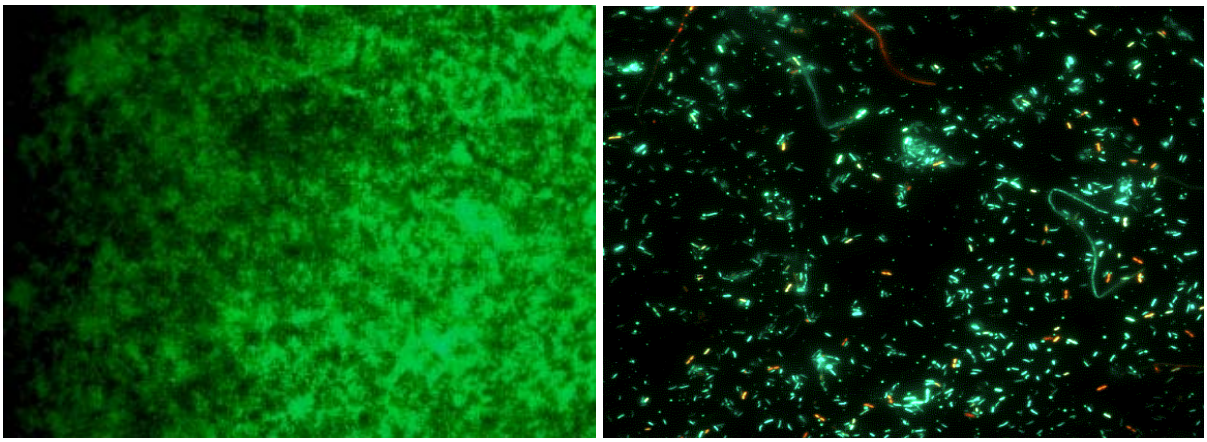

Figure 4. Stained D. *vulgaris* biofilm at 168 hours. Viewed under oil immersion objective (Total magnification of 1000x) using epifluorescent microscopy.

CONCLUSIONS

Tests using the fractionated pepper extracts are progressing rapidly. More needs to be done. The gas chromatography analysis on a number of fractions is underway. The involvement of other microorganisms in the tests will be increased. The results of these fractions and their interactions will commonly used pipeline chemicals will be in the future reports.

Results on the use of foams to deliver the biocides will also be in future reports. The various surfactants available and the effects of a range of pH will be given.

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