AN ANALOGUE MIC SYSTEM WITH SPECIFIC BACTERIAL CONSORTA, TO TEST EFFECTIVENESS OF MATERIALS SELECTION AND COUNTERMEASURES

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ABSTRACT

A standardized system has been established wherein microbes were recovered from specific sites (waters, sediments, slimes, tubercles) and at least six physiologically different groups of microbes were characterized and identified. These organisms were selected for properties known to be associated with microbial influenced corrosion (MIC), such as slime formation, acid production, sulfate reduction, iron chelation and precipitation, and hydrogen utilization. The organisms were characterized by their fatty acid composition. Coupons of the test material or surface treatment were embedded in epoxy, and utilized as working electrodes. The sterilizable test system was designed to contain multiple working electrodes, a titanium counter electrode, and a salt bridge designed to hold a standard calomel electrode. After sterilization, site water, or its surrogate enriched with dilute microbiological medium, was introduced into the system, and inoculated with a mixture of the isolated microbes. The polarization resistance (R_p) and open circuit potential (OCP) were compared to sterile controls, using electrochemical impedance spectroscopy (EIS) and electrochemical noise (ECN). Throughout the test, test metals were recovered, and the microbial community composition and metabolic activity of the organisms on the metal surface were determined. Biomass was determined by direct count of stained microbes, and by the total ester-linked fatty acids. Community composition was determined by plating and counting colony forming units (CFU's). Isolates were identified by morphologic properties, and by analysis of the polar lipids using gas chromatography/mass spectrometry. Microbial biofilm metabolic activity was determined with labeled acetate incorporation into the lipids. Biofilm microbial morphology was determined by scanning electron microscopy (SEM). In this report, the test system was used to examine the effects of sublethal and lethal exposure to hypochlorite on bacteria, which induced tubercles on mild steel samples.

INTRODUCTION

MIC is being increasingly recognized as a serious problem when surfaces are exposed to natural waters[1]. Where localized corrosion is associated with tubercles, slimes, discolorations, odors of anaerobic metabolism, sludges, "under deposit corrosion", which are associated with pitting corrosion, microbial involvement is indicated. Localization of corrosion to welds, heat affected zones (HAZ), areas of stagnation, dependent areas, where water could collect, reinforces the contention that MIC is involved. Certain metalographic "signatures" suggest MIC. Specific attack of austenitic or ferritic components of welds[2] or "tunnel" pitting of pipe line steel[3] strongly suggests bacterial involvement.

We have frequently been asked to provide consultation on the selection of materials for replacement of MIC damaged systems. How can rational decisions be made in the short time spans that are available before the replacement material must be ordered?

This paper describes a technique whereby microorganisms with known MIC propensities can be recovered from a specific site, their physiology defined, and phenotypic identification determined. These microbes can be utilized in consortia to test the relative resistance of specific candidate materials to MIC, in a test system where the actitivity of the microbial consortia can be increased by incorporation of dilute nutrients. The nutrient can be added to water from the specific site, or a surrogate can be created in the laboratory. The water or medium is pumped into a sterile system containing the test materials as working electrodes. The system is usually operated in the flowing condition with a dilution rate of about 10-20% of the total test system volume removed per hour. This test system can provide an acclerated response of specific materials or surface treatments to MIC. The corrosion rates of the test materials are monitored using non- distructive electrochemical methods. Polarization resistance is derived from measurements of electrochemical impedance spectroscopy (EIS). Open circuit potential (OCP) and electrochemical noise (ECN), which may be indicative of pit formation, can also be analysed. To document that the microbes are involved there must be distinctive differences in the electrochemical signal between the inoculated flasks and the sterile controls, and the microbes must both be recoved from the working electrode surface and shown to be metabolically active. The test system allows the examination of the activities of specific combinations of microbes against different materials or surface treatments. The effectiveness of chemical or mechanical countermeasures can be tested with this system and the optimization of treatment scheduling established.

The test system can involve the acceleration of the microbial activity on the working electrode surfaces, so that responses to different test materials, surface treatments, or countermeasures are compared in a reasonable time. To be validated, long term tests of coupons placed in side stream devices subjected to the ambient conditions at the particular location must be a part of the testing program.

EXPERIMENTAL PROCEDURES

Recovery of the microbes

Water samples, slimes, sediments from MIC damaged components were collected in sterile plastic containers and shipped, on ice by air, to the laboratory for isolation within 24 hours of sampling. In the laboratory, the microbiota in water samples were recovered from sterile polycarbonate filters using a tangential flow filter apparatus. Tubercles, broken with a sterile scalpel, were scraped into sterile test tubes containing dilute medium and glass beads. The contents were vortexed under a stream of nitrogen, and serial diluted in sterile media.

On completion of the EIS tests an open glass extraction vessel was clamped to the metal samples, using an o-ring seal. The biofilm was resuspended into 0.5 ml of sterile medium, using a sterile point sonicator[4]. The suspension was serial diluted into sterile tubes and plated on agar medium or inoculated into anaerobic most probable number dilution tubes (MPN) for enumeration of aerobic and anaerobic bacteria, respectively. Samples were also diluted and stained with acridine orange for direct microscopic counts. All operations are performed in laminar flow hoods.

Isolation of the microbes

The dilution tubes obtained from the recovery procedure were streaked on Winogradski's agar medium, containing Hutner's mineral salts. The media were designed to select for various microbial physiological groups. Phenol red was added to the media to detect acid producing bacteria. Ferrous ammonium citrate was added to detect iron precipitating bacteria. For anaerobic medium, ferrous sulfate, 0.25mg/l) was added to detect sulfate reducing bacteria (SRB). Isolates were purified by repeated picking and restreaking of colonies until only one colony morphology was obtained. Individual bacterial isolates were then screened using gram stain, oxidase tests, catalase tests, and the Miniaturized Microorganism Differeniation System (Minitek) system.

Characterization of the bacterial isolates

Isolated bacteria were grown using solidified media (Winogradski's, with Hutner's salts and trace metals [5]. Their total fatty acid composition was measured after saponification in methanol to form the methyl esters and analysis by capillary gas chromatography using the Microbial Identification System consisting of a Hewlett-Packard 5980A capillary gas chromatograph, autosampler, computer with the microbial identification and library generation system (Microbial ID, Inc, Newark DE).

Sample Preparation

Coupons of the test metals, 16 mm in diameter, were finished to a 600 grit finish and embedded in epoxide, so only one surface was exposed to the solution. The edges were coated with additional epoxide, under microscopic control. Counter electrodes of titanium, 41mm x 147mm, bent in a "U" shape to surround the working electrode, were prepared by connecting coaxial cable

to the titanium with solder and covering the solder and copper with epoxy, so only the titanium is exposed to the solution. Standard calomel electrodes, connected to the working solution via salt bridges fitted with Vicor tips, served as reference electrodes. The surfaces were cleaned with ethyl alcohol. In some experiments a passive film was generated on the surface of the metal. For example, some of the mild steel surfaces were treated 1% (w/v) sodium nitrite, at room temperature.

Test Apparatus

The electrochemical test cell was made from a 500ml glass kettle with an o-ring seal at the top (Figure 1). The kettle was covered with a polypropylene disk, 1 cm thick, through which leads for the multiple working electrode unit, the salt bridge, the counter electrode, the inoculation port, the medium entrance drip tube, and the exit ports were placed. A viton o-ring fitted in a groove at the top of the kettle, and the cover is clamped to the kettle with "C" shaped clamps. The medium inlet drip tube had an air space to prevent back contamination. Ventilation ports for the drip tube and the kettle were connected to filter holders containing 0.2 um pore diameter filters.

To expose additional coupons, used for recovery of microbes from the biofilms, and for measurements of microbial activities, a second test cell was connected in parallel with the electrochemical cell (Figure 2). This apparatus, fabricated from a tall one liter graduated beaker, had the same type of drip tube inlet and exit. Coupons encased in epoxy resin were suspended from a polypropylene ring, with stainless steel hooks. The use of the ancillary vessel allowed samples to be recovered during the test period without disturbing the electrochemical test cell. Both vessels were mixed with a magnetically driven, teflon coated stir bar.

The apparatus was assembled with double coverings of aluminium foil over each port. The apparatus was sterilized by exposure to an atmosphere of ethylene oxide/carbon dioxide 80/20 for 4 hours at 50 C. It is important to be sure that the gas sterilization does not effect the coupon surface. Wet steam autoclaving can induce corrosion on mild steel coupons. The apparatus was then connected to Masterflex peristaltic pumps using silicon tubing. The medium was prepared in 45 liter glass carboys and autoclaved at 120 C for 3 hours. It was allowed to cool with air equilibration through a 0.2 um microbiological filter. Medium was delivered through the drip tubes to the vessels at a rate 60 ml/hr (15% replacaement of the vessel volume/hr). The medium was removed from the vessels using masterflex pumps calibrated to deliver 100 ml/hour into sealed waste vessels. The apparatus was operated in a laminar flow hood. Additional ports were provided for biocides.

Electrochemical Analysis

EIS analysis was performed using the Solartron 1286 electrochemical interface and 1250 frequency response analyzer controlled by a microcomputer. Sinusoidal potentials of 5 mV (rms) ranging from 5 mHz to 10 Hz were applied to the working electrode. In preliminary studies, these frequencies were found to give the most information in the least amount of time, for these

particular tests. The phase shifts of the resulting currents were plotted either as the immaginary impedance versus the real impedance in a Nyquist format, or as the log of real impedance versus the log of the frequency in a Bode plot. The system was monitored with a dual channel oscilloscope to verify the waveform of the perturbations. Electronic switching between three electrode systems was achieved with a Keithly scanner. Open circuit potential (OCP) and electrochemical noise (ECN) were measured with a Solartron 7090 precision voltmeter. Small amplitude cyclic voltametry (SACV) was also preformed.

Microbial Metabolic Activity

The test coupons recovered from parallel apparatus (Figure 2) were immediately covered with a modified 60 ml seperatory funnel containing a flanged base with a pre-extracted Kalrez o-ring. The apparatus was clamped to the disk so only the biofilm is exposed. One-half ml of medium without glucose, containing 2.5 mCi ^{14}C acetate, was added to the clamped vessel, the samples were shaken vigorously, and incubated for 30 min at room temperature. Zero time controls and the 30 min incubations were terminated by the addition of the chloroform-methanol extraction mixture (see below). After at least 2 hours, additional chloroform and water were added to form a two phase system and the lipids recovered in the chloroform phase [6]. The lipids for community structure analysis were extracted from other coupons without exposure to the labeled precursors.

LIPID EXTRACTIONS

The analytical sequence utilized for phospholipid ester-linked fatty acid (PLFA) analysis involved extracation, fractionation on silicic acid, derivatization, and analysis by capillary gas chromatography (GC) with mass spectral structural confirmation. The modified one-phase Bligh and Dyer extraction was utilized for all samples [6]. Triplicate samples were extracted separately and all data is expressed as the mean of three determinations unless otherwise stated. After the overnight separation of the lipid and aqueous phases in the second stage of the extraction, the organic fraction was filtered through fluted Whatman 2V filters pre-extracted with chloroform and reduced in volume using a rotating evaporator. The samples were transferred to teflon lined screw capped test tubes and dried under a stream of nitrogen at room temperature.

DETERMINATION OF RADIOACTIVITY

Radioactivity was determined after drying the sample in a scintillation vial, bleaching with benzolperoxide in chloroform, redrying, and adding Aquasol scintillation cocktail. Radioactivity was determined using the LKV scintillation spectrometer. Quenching was controlled using commercially prepared standards and the channels ratio method [7].

POLAR LIPID ISOLATION

Silicic acid columns were prepared using 1 g Unisil (100-200 mesh,

Clarkson Chemical Co., Inc., Williamsport, PA) activated at 100°C for 60 min and pre-extracted with chloroform. Columns were prepared with the approximate ratios of 50:1 stationary phase to lipid (dry weight) and 1.7:1 stationary phase column bed height:cross sectional area. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 10 ml of chloroform, acetone, and methanol eluted the neutral, glyco-, and polar lipids. The polar lipid fraction was dried under a stream of nitrogen.

The radioactive samples were recovered in chloroform and fractionated using disposible silicic acid columns (0.5 g) made from Pasteur pipettes using the solvent sequence described[7].

MILD ALKALINE METHANOLYSIS

The mild alkaline methanolysis procedure was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids [6].

DETERMINATION OF MONOUNSATURATED PLFA DOUBLE BOND POSITION AND CONFIGURATION

The dimethyl disulfide (DMDS) adducts of mono-unsaturated PLFA were formed to determine double bond position and geometry using methods previously described [8]. Samples were analyzed by capillary GC and GC/MS as described below. These derivatives increase the resolution between cis and trans geometrical isomers in capillary GC.

GAS CHROMATOGRAPHY

Dry fatty acid methyl esters (FAME) were dissolved in isooctane containing the internal standard of methyl nonadecanoate. Samples of 1.0 ul were injected onto a 50 m nonpolar, cross-linked methyl silicone fused silica capillary column (0.2 mm i.d., Hewlett Packard) in a Shimadzu GC-9A GC. A 30 s splitless injection with the injection temperature at 270°C was used. Hydrogen at a linear velocity of 35 cm/s was the carrier gas with a temperature program starting with an initial temperature of 80°C. After a one minute delay at 80°C, the temperature was programmed at a 10°C rise/min to 150°C, followed by a 3°C/min to 240°C, followed by a 5°/min rise to 280°C with a terminal isothermal period of 5 min at 280°C. Detection was by hydrogen flame (F.I.D.) using a 30 ml/min nitrogen make up gas at a temperature of 290°C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Nelson Analytical 3000 Series Chromatography Data System (Revision 3.6) [9].

GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS).

Tentative component identification prior to GC/MS analysis was based on comparison of the retention time data with data for standards from Supelco, Inc. (Bellefonte, PA) and Applied Science Labs, Inc. (State College, PA) and previously identified laboratory standards. FAME were tentatively identified by co-elution with authentic standards supplied by Supelco, Inc. (Bellefonte, PA) and Applied Science Labs., Inc. (State College. PA) or previously

identified laboratory standards. GC/MS analysis was performed on a Hewlett Packard 5996A GC/MS fitted with a direct capillary inlet utilizing the same chromatographic system except for use of a helium carrier gas and the temperature program which was begun at 100°C and increased to 280°C at 3° C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1800 and 2000 V, the transfer line maintained at 300°C, the source 280°C and analyzer 250°C, and the GC/MS was autotuned with DFTPP (decafluorotriphenylphosphine) at m/z 502 with an ionization energy of 70 eV. The data was acquired and manipulated using the Hewlett Packard RTE 6/VM data system. Other conditions were as described previously [9].

For certain experiments when higher sensitivity was required the VG-Trio 3 triple quadrupole mass spectrometer was utilized with chemical ionization with detection with either negative or positive ions.

FATTY ACID NOMENCLATURE

Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond nearest to the aliphatic (w) end of the molecule indicated. This is followed by the suffix c for cis and t for trans configuration of monoenoic fatty acids. The prefixes i, a, or br indicate iso, anteiso, or branched (position undetermined). Mid-chain branching is indicated by the number of carbon atoms from the carboxyl end of the molecule and Me for the methyl group (10Me16:0 is a 17-carbon PLFA). Cyclopropane rings are indicated with the prefix cy with the ring position indicated from the methyl end of the molecule.

RESULTS

BACTERIA UTILIZED IN MIC EXPERIMENTS

Colonies of bacteria isolated after dilution in appropiate media from MIC tubercles from mild steel piping in utilities were used to characterize six physiological types of bacteria. They were a gram-positive spore forming bacillus, an iron reducing bacteria, an iron precipitating strictly aerobic bacteria that produced copious slime, an acetic acid producing facultative anaeraobe with a rapid growth rate at pH 8.5, and a second acetic acid producing facultative anaerobe. Each was isolated by aerobic incubation. The sixth bacterium was a lactate-utilizing, hydrogenase positive, sulfate-reducing anaerobic bacteria. Each of the five aerobic bacteria had a distinctive colony morphology on culture using solidified Winogradski's medium, so they could be identified, and the CFU of each bacterium determined simultanaeously in recovery experiments. The anaerobic sulfate-reducing bacteria (SRB) were enumerated using three tube MPN reactions.

EFFECT OF THE BACTERIAL CONSORTIA ON THE CORROSION

The inoculation of the six strains into the test vessels induced visible tubercles on the C1020 mild steel coupons in three days. The polarization resistance (R_p) was 800 ohms for steel exposed to bacteria, compared to 2400 ohms in the sterile control. The 800 ohms corresponded to an I_{corr} of 19.7

uA/cm^2, compared to an I_{corr} of 4.6 uA/cm^2 for the control. Tafel constants were measured using cathodic polarizations. The R_p was measured using EIS.

The presence of the microbial consortia on the metal surface induced a different corrosion response. In the sterile controls an exposure to the flowing medium induced the formation of a corrosion film on the surface of the sterile electrode (Figure 3). When the friable rust layer was removed the surface of the mild steel was relatively smooth (Figure 4). When the vessels were inoculated with the six physiological types of bacteria, and the system allowed to develop for 3 days, a mat of bacteria covered the surface (Figure 5). Under the biofilm and the corrosion products, the surface of the mild steel shows a marked pitting response (Figure 6). MIC tends to induce localized corrosion, so the increased corrosion was in fact much more serious.

EXPERIMENTAL TEST OF BIOCIDE EFFICACY

The test system was utilized to test the effects of countermeasures in the control of MIC. In a test of biocide efficacy against a biofilm in tubercles on mild steel, the system was allowed to incubate, after inoculation with bacteria for 4 days. At this time, there was a distinct difference between the corrosion rate of the inoculated test systems and the sterile controls. At this point, one set of vessels was treated with sodium hypochlorite at a concentration of 2 ppm active chloride residual for one hour, followed in 24 hours with a second exposure for 2 hours. This was a sublethal exposure. A second set of vessels was treated with a slug dose of 16 ppm active chloride residual for two hours followed by a 24 hour exposure to 2 ppm (residual). This induced a lethal exposure. Both were then allowed to recover for 24 hours in the absence of biocide.

The sublethal exposure resulted in a temporary increase in the polarization resistance of 25 and 55% during the exposure. This was followed by a rebound period in which the polarization resistance increased 1.9 fold faster than the untreated control. An increase in polarization resistance corrospondes to a decrease in corrosion rate.

The recoverablity of the microbes from the exposed coupon surfaces showed remarkable differences between the lethal and sublethal exposures to biocide (Table 1). The sublethal exposure showed essentially no depression in the recoverability of viable bacteria of each of the six physiological types of bacteria. The results were essentially identical to the untreated control. The untreated biofilm in the course of the two day period resulted in an approximate doubling of the total microbial community. The planktonic community showed a decrease in some of the bacteria of 1-2 orders of magnitude during the exposures.

The lethal exposure however resulted in the depression of the recoverable viable bacteria, to two orders of magnitude in the relatively resistant gram-positive Bacillus and three to four orders of magnitude for the more sensitive bacteria. The 24 hours recovery did not allow for complete restoration of the biomass to pre-treatment levels. The planktonic community showed a complete loss of viable recoverable cells for the total period of the HOCl exposure. This was also observed as a clearing of the

inoculated test vessels. The numbers of planktonic bacteria returned to pretreatment levels after the 24 hour recovery period, except for the SRB, which remained about an order of magnitude below the pre-treatment level.

The biomass of the cells on the exposed surfaces as determined by the acridine orange direct microscopic counts showed essentially no differences between the treated and control coupons. This technique counts nonviable as well as viable bacteria.

The morphology of the biofilm after the sublethal treatment (Figure 7) showed essentially no difference from the untreated control (Figure 5). The biofilm from the lethal treatment showed only corrosion products on the surface and few bacteria (Figure 8). Scanning electron micrographs of the biofilms after sublethal exposure show damaged cells as amorphous materials amongst the adherent bacterial biofilm. Removing the corrosion products from the coupon, subjected to lethal treatment, displays localized pitting corrosion (Figure 9).

The most sensitive measure of biocide effects was on the activity of the microbes in the biofilm. The activity was measured as the rate of lipid biosynthesis from 14-C-labeled acetate. The sublethal exposure to HOCl resulted in a depression of the rate of lipid synthesis by 55% for the first sublethal exposure and by 40% for the second sublethal exposure. The 24 hour recovery period resulted in a "rebound" in which the rate of lipid synthesis was 40% greater than the untreated control. The untreated control showed about a 20% increase in activity during the experiment.

With the lethal exposure to HOCl the lipid biosynthetic of the biofilm was depressed to undetectible levels throught the total period of treatment. During this period the untreated control showed a doubling in lipid biosynthetic activity. After the 24 hour recovery period the treated biofilms had an activity roughly half the untreated levels which was approximately 20% greater than the initial activity.

DISCUSSION

NONDESTRUCTIVE MONITORING OF MIC

In accelerated corrosion tests it is important to monitor the corrosion over the period of the test. This requires a monitoring system that is non-destructive to the biofilm. EIS, OCP, ECN, and small amplitude cyclic voltametry (SACV) have been shown to work effectively in monitoring MIC coupons [10]. The EIS analysis allows determination of both the solution resistance and the polarization resistance (Figure 10). Knowing the polarization resistance the value for I_{corr} can be estimated from the Stern-Geary equasion. The DC polarization measurements distroy the biofilm. Thus, the multiple electrode system described was useful, not only for replicating the response within the system, but also for obtaining the Tafel Parameters. Repeated measurements of R_p by EIS on noble electrodes with biofilms shows little evidence of damage to the biofilms. OCP is an even less perturbing measurement. Borenstein and White [12] in this symposium have used OCP to document MIC on weldments. OCP can be utilized to indicate the appearence of

pitting in stainless steel weldments [11].

GROWTH OF BACTERIAL BIOFILMS

The degree of the MIC pitting corrosion appears to be somewhat dependent on the growth rates and activities of the microbial biofilms. Consequently in test systems, the microbiota should be in the same relative nutritional status as they are usually found in nature. The usual condition of microbial biofilms in nature is in a semi-starved status [13]. In the test systems reported herein, a medium with a 50 mg/l carbon content consisting of 20 mg/l glucose, 10mg/l lactate, 10mg/l citrate, 5 mg/l of yeast extract and 5 mg/l peptone with minerals, salts and 1mM Tris buffer pH 8.5 was delivered to the test vessels at a rate of 10% of the 500 ml test vessel volume/hour. This nutrient content and turnover produces a suboptimal planktonic microbial density of about 10^8 cells/ml when compared with richer media. Growth rates in medium made at 10 times the concentrations used in the test systems (500 mg carbon/l) resulted in bacteial doubling times of between 3 and 8 hours. With the lower density of nutrients and the 10 hour residence time the test system was thus maintained at suboptimal growth rates for planktonic cells. The organisms in the biofilm were not subject to washout. The nutritional status of these organisms can be determined by analysis of the extracatible lipids. High ratios of the endogenous storage lipid polymer, poly beta-hydroxy alkanoate (PHA) to the PLFA correlate to nutritional imbalance [14] and the ratio of the rates of formation PHA/PLFA can be readily determined by the ratioactive assay used in these experiments [7]. The presence of specific component PLFA also have been shown to correspond to conditions of nutritional imbalance [9,15,16]. Nutritional imbalance in the biofilm microbiota can be detected in the PLFA patterns of relatively high proportions of the 17 and 19 carbon cyclopropane PLFA and the high proportions of the trans isomer of the 18 carbon monoenoic PLFA (Table 2).

BIOMASS OF MICROBES ON THE CORROSION COUPONS

The biomass of the microbiota on the corrosion coupons based on acridine orange direct counts (AODC) correlated directly with the estimates based on the total PLFA recovered from the coupons. Assuming that 8×10^{12} bacterial cells correspond to 100 umoles PLFA [17] the roughly 28 nmoles PLFA correspond to the 5×10^9 cells detected by AODC in the untreated controls or the biofilms after recovery.

RECOVERY OF MICROORGANISMS FROM THE CORROSION BIOFILMS

The data of Table 1 illustrate that the inoculation of a mixed group of organisms into the bulk fluid of a test system initiates the colonization of the metal surfaces exposed to that fluid. Even the absolutely anaerobic SRB were able to survive inoculation in aerated fluids and find anaerobic microniches created by the metabolic activities of the aerobic heterotrophic bacteria in the inoculum and to proliferate on the coupon. The analysis of the PLFA recovered from the biofilm on the surface shows clearly that the "signature" biomarkers of the SRB's, the i17:1 PLFA were readily detected. The PLFA from the other members of the consortia were also readily

detectible.

EFFECTS OF SUBLETHAL AND LETHAL EXPOSURE TO BIOCIDE

The test system provided a mixed community microbial biofilm that induced tubercles, typical of MIC, rapid corrosion, and the appearance of pits. Sublethal exposure to HOCl resulted in little change in the recoverable sessile bacteria but demonstrable changes in the activity of the biofilm. These changes resulted in changes in corrosion rates measured by EIS. Removal of the biocide resulted in a rapid "rebound" in which the bacterial activity exceeded the untreated control, and the MIC was stimulated. Lethal exposure resulted in loss of the microbes from the biofilm and complete inhibition of the metabolic activity during the test period. The different types of microbes showed a differential response to the biocide treatment. With termination of the biocide exposure the community regrew on the coupons even though the presence of viable bacteria in the bulk fluid was not detectable.

CONCLUSIONS

1) A system for accelerated test of the response of materials, surface treatments of materials, and countermeasures was examined in the presence of a microbial consortia formed from organisms isolated from a specific site.

2) Microbes inoculated into sterile test system were able to colonize working electrodes. The biofilm was created with community structures and nutritional states to match those of the systems whose behavior was modeled.

3) The non-destructive EIS technique gave values of polarization resistance and solution resistance. Thus, an estimation of the corrosion rate could be obtained in the low ionic strenghth medium, without destroying the biofilm.

4) The corrosion rates was correlated to the metabolic activity of the microbes on the coupon surface, and the effects of countermeasures were examined directly.

5) The microbes on the working electrode were recovered and tested for viability. Shifts in the biofilm microbial community structure, nutritional status and metabolic activity could be examined for effects on corrosion. Viability by recoverability and enumeration by CFU was compared to the recovery of signature biomarker PLFA.

6) The test system defined in this paper offers the capacity to make rational decisions regarding the suitability of specific materials for a specific application with the expousre to a particular group of microbes.

7) This test system allows for directly testing various welding technologies or surface treatments against specific microbial consortia under defined conditions. The direct examination of the actual microbial community structure and specific microbial activities can be readily correlated to

non-destructive corrosion monitoring.

8) This test system can be utilized to define the effectiveness of countermeasures as illustrated by the example in the paper. In this way, optimal biocide selections, mixtures, concentrations, and treatment protocols can be tested against the materials and the microbes from a specific location. The community structure, nutritional status, and metabolic activity of the sessile microbiota that actually influence the corrosion can be directly manipulated and monitored.

9) The technology lends itself to further refinements such as the utilization of mass labeled precursors (13-C, 15-N) and the recovery in the specific "signature" biomarkers from microbes localized in specific portions of the corrosion coupon (such as the pits). This becomes possible because of the advances in sensitivity and resolution of gas chromatography/ mass spectrometry.

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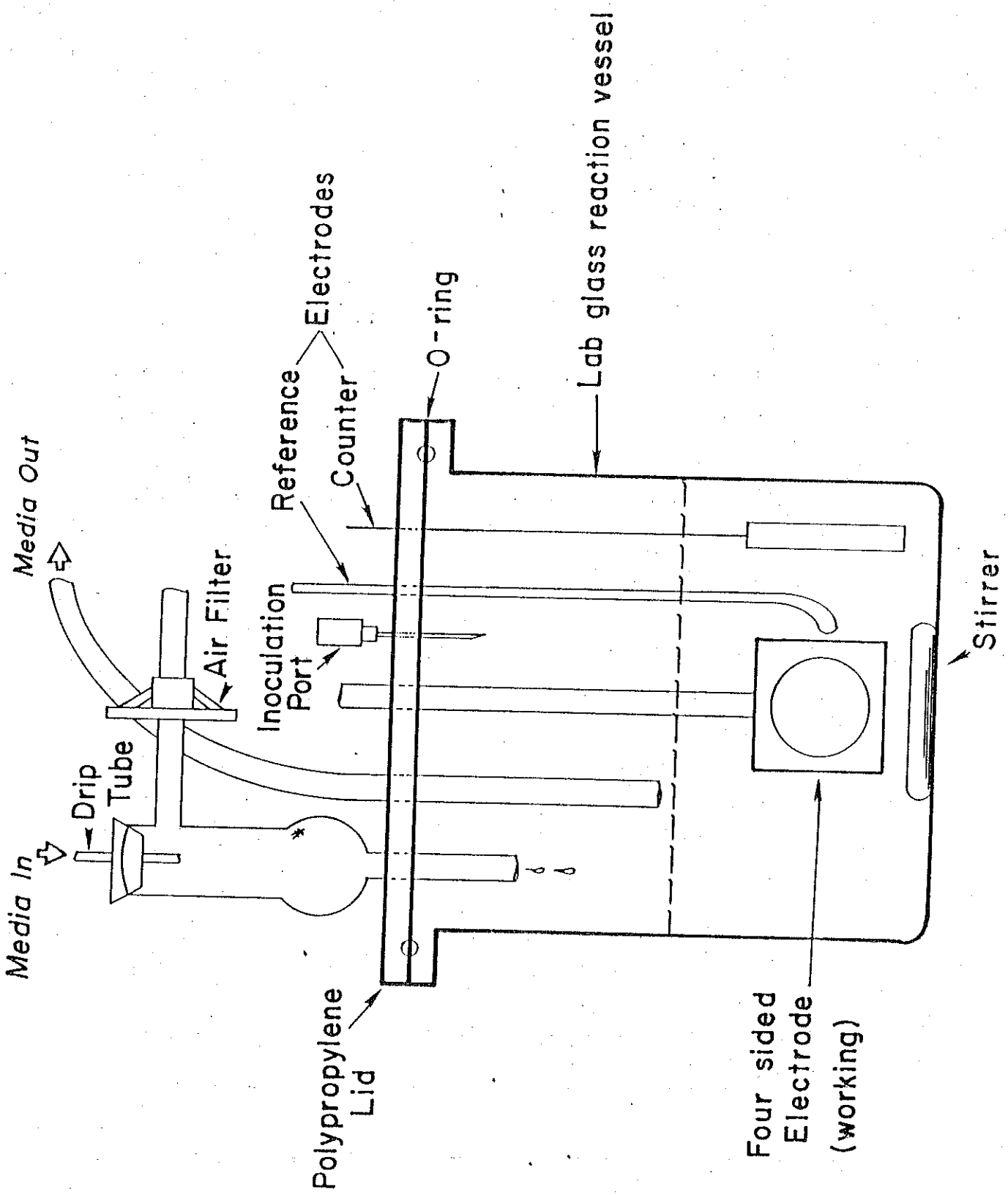


Figure 1. Experimental test vessel for electrochemical analysis of accelerated corrosion. Both the media in and media out are connected to reservoirs with Masterflex pumps and silastic tubing. The four sided electrode can be rotated so each face of the working electrode can be placed adjacent to the reference electrode during the electrochemical analysis.

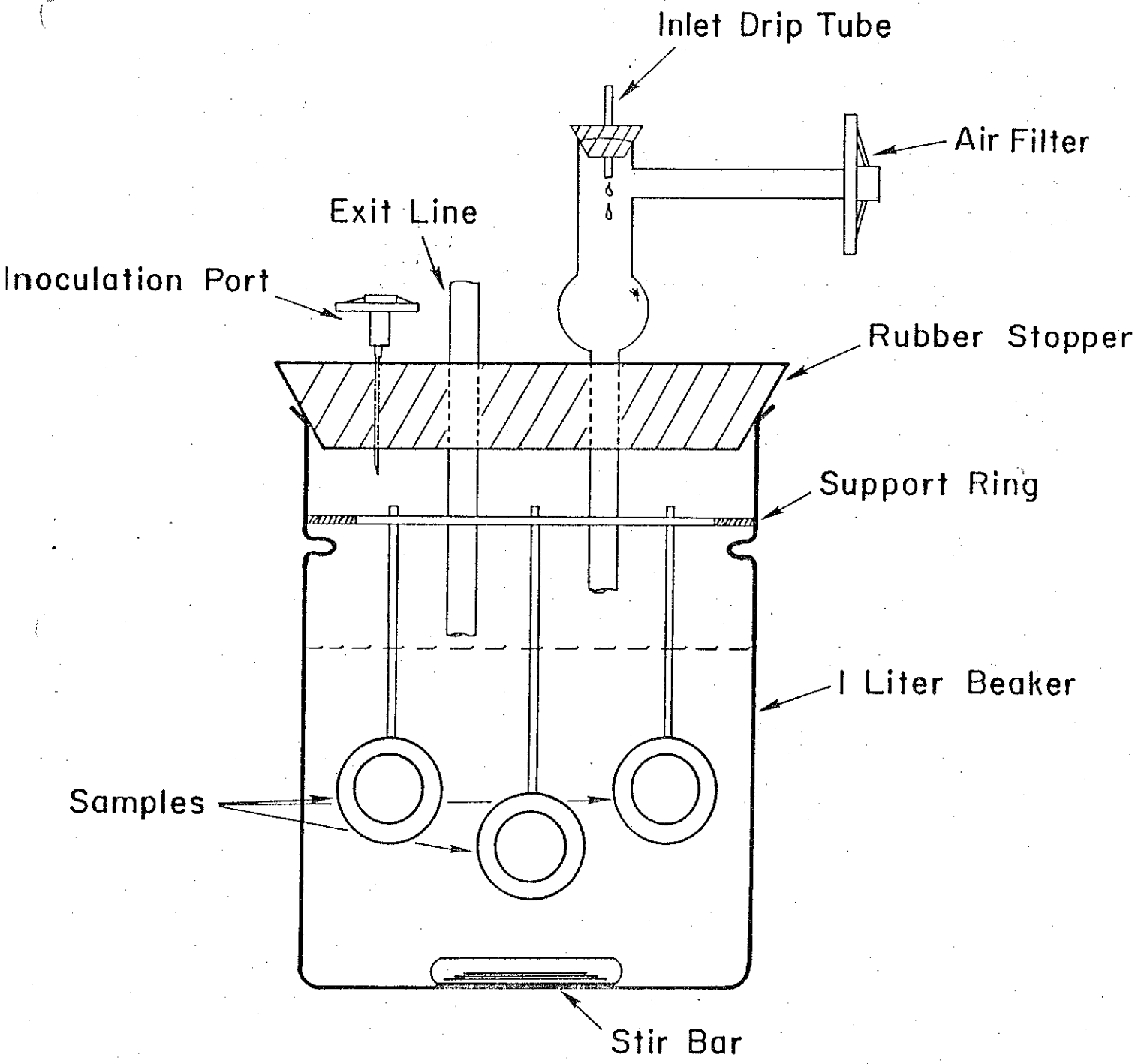


Figure 2. Ancillary test vessel connected in parallel with the electrochemical test vessel shown in Figure 1. The samples embedded in epoxy resin are suspended from a nylon support ring using 316L stainless steel hooks.

Figure 3. Scanning electron micrograph of the corrosion products associated with the sterile, mild steel coupon induced after three days of exposure in the test systems. Magnification of 1000X.

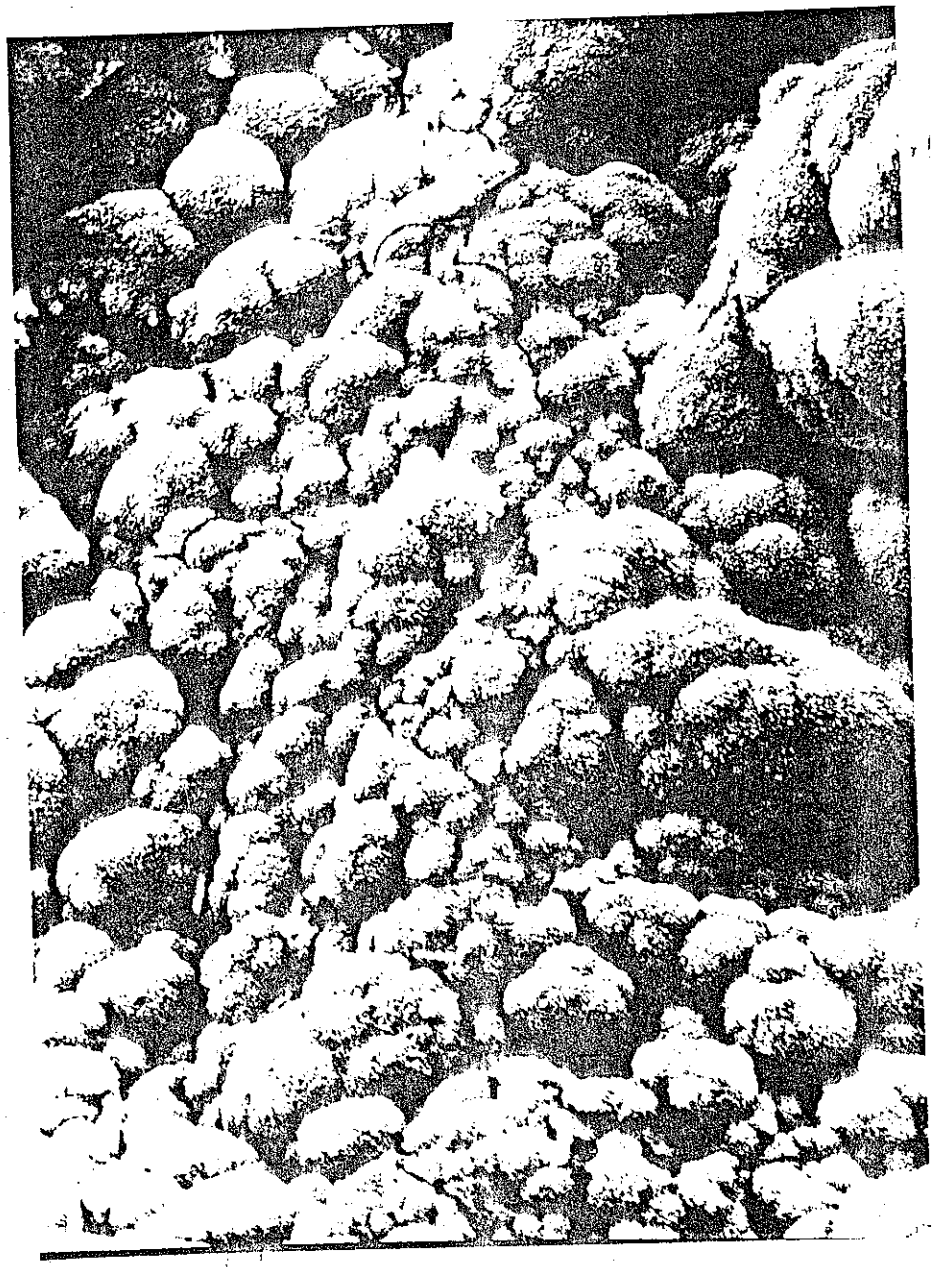


Figure 4. Scanning electron micrograph of the metal surface in the sterile after removal of the friable corrosion products shown in Figure 3 with a sterile spatula. Magnification is 1000X.

Figure 5. Scanning electron micrograph of the biofilm formed on mild steel coupons 3 days after inoculation with the six physiological types of bacteria associated with MIC. Magnification is 4000x.

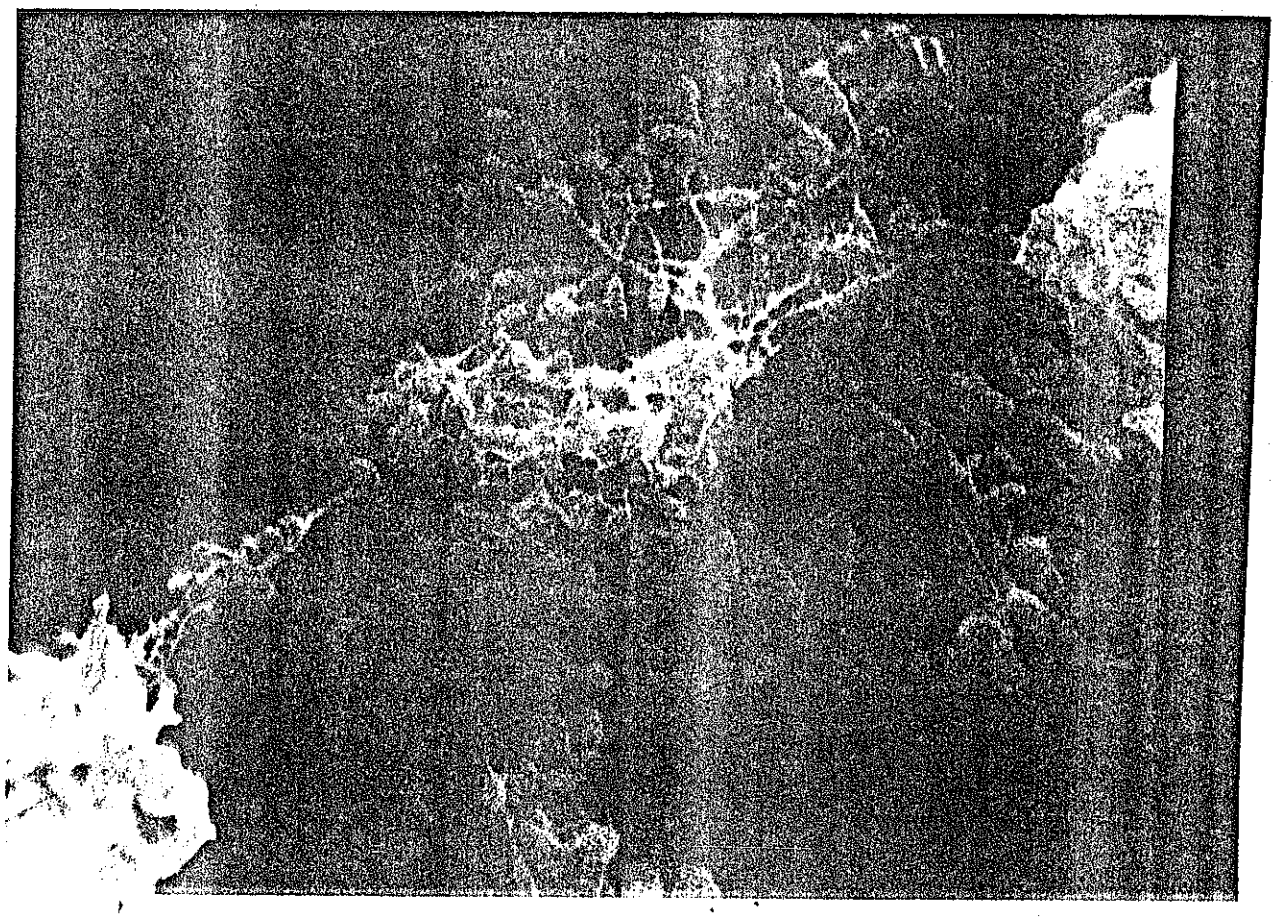


Figure 6. Scanning electron micrograph of the mild steel surface after removal of the corrosion products and biofilm from the surface illustrated in Figure 5. Magnification is 1000x.

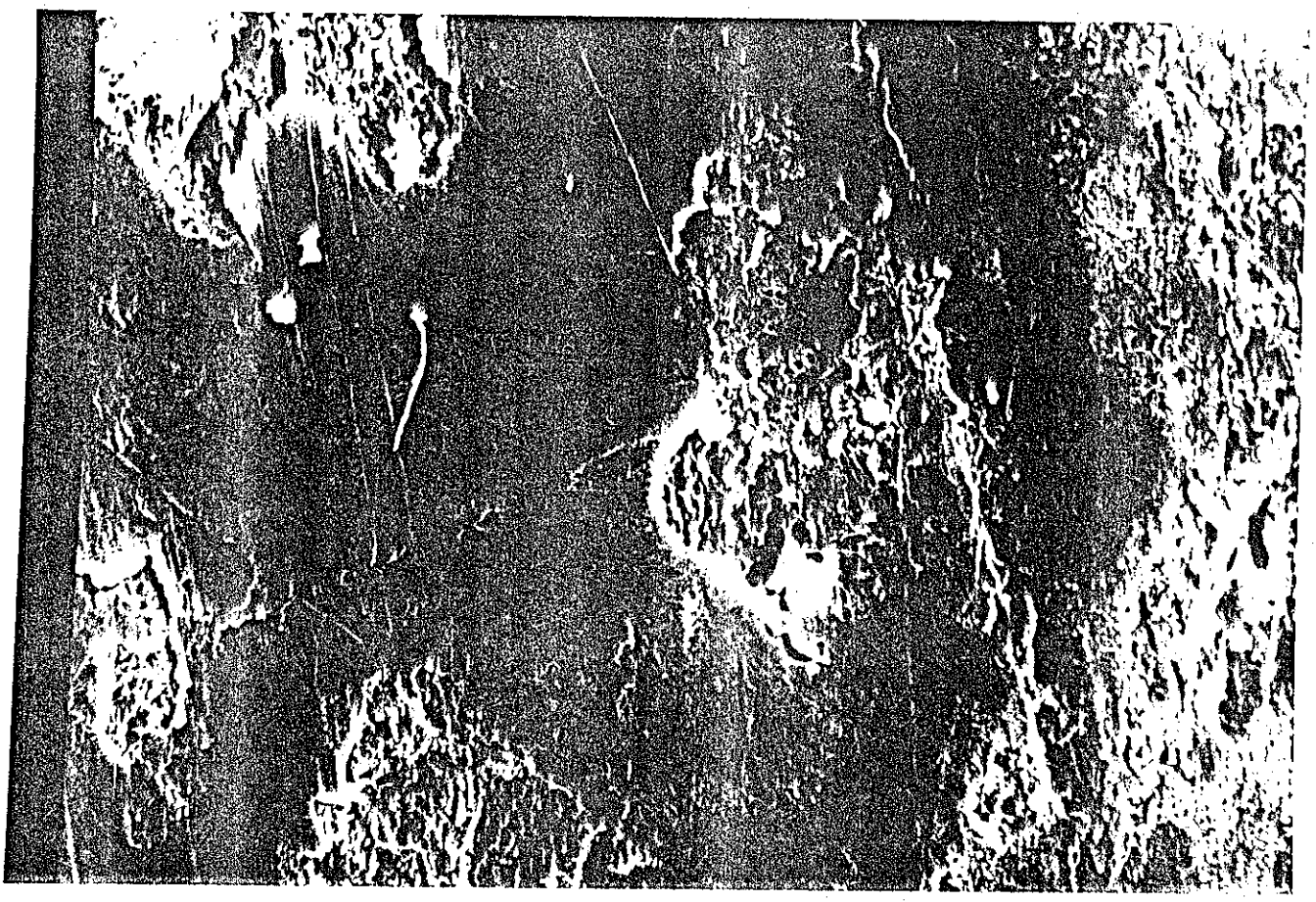


Figure 7. Scanning electron micrograph of the surface of mild steel coupon after exposure to sublethal (2 ppm HOCL at pH 8.5 for 1 hour, followed by 2 ppm for 2 hours the next day). Magnification is 4000X.

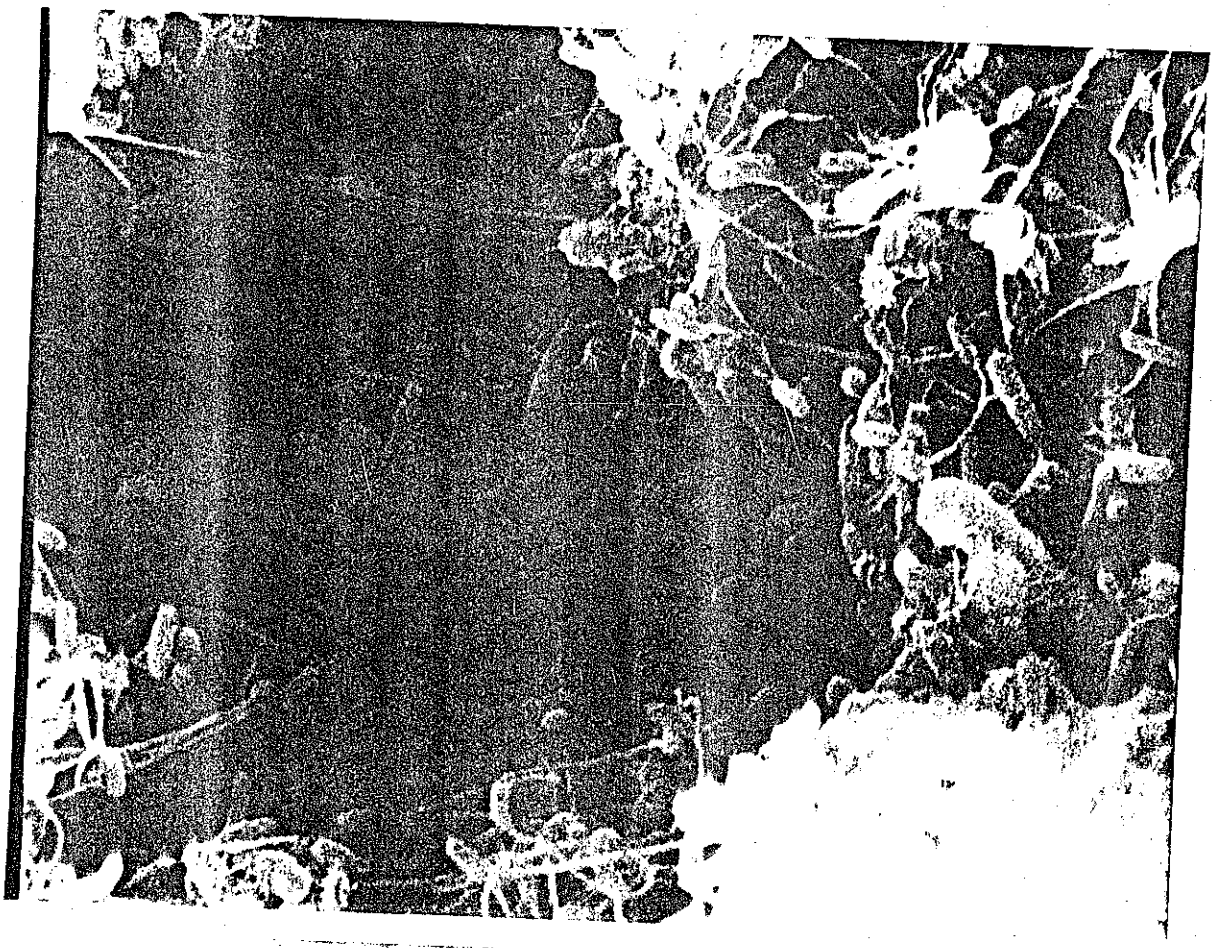


Figure 8. Scanning electron micrograph of the surface of mild steel coupon after a biofilm pictured in Figure 5 was exposed to 16 ppm (residual) HOCl for 2 hours followed by 2 ppm (residual) for 24 hours. Most of the bacteria have disappeared from the tubercle. Magnification is 4000X.

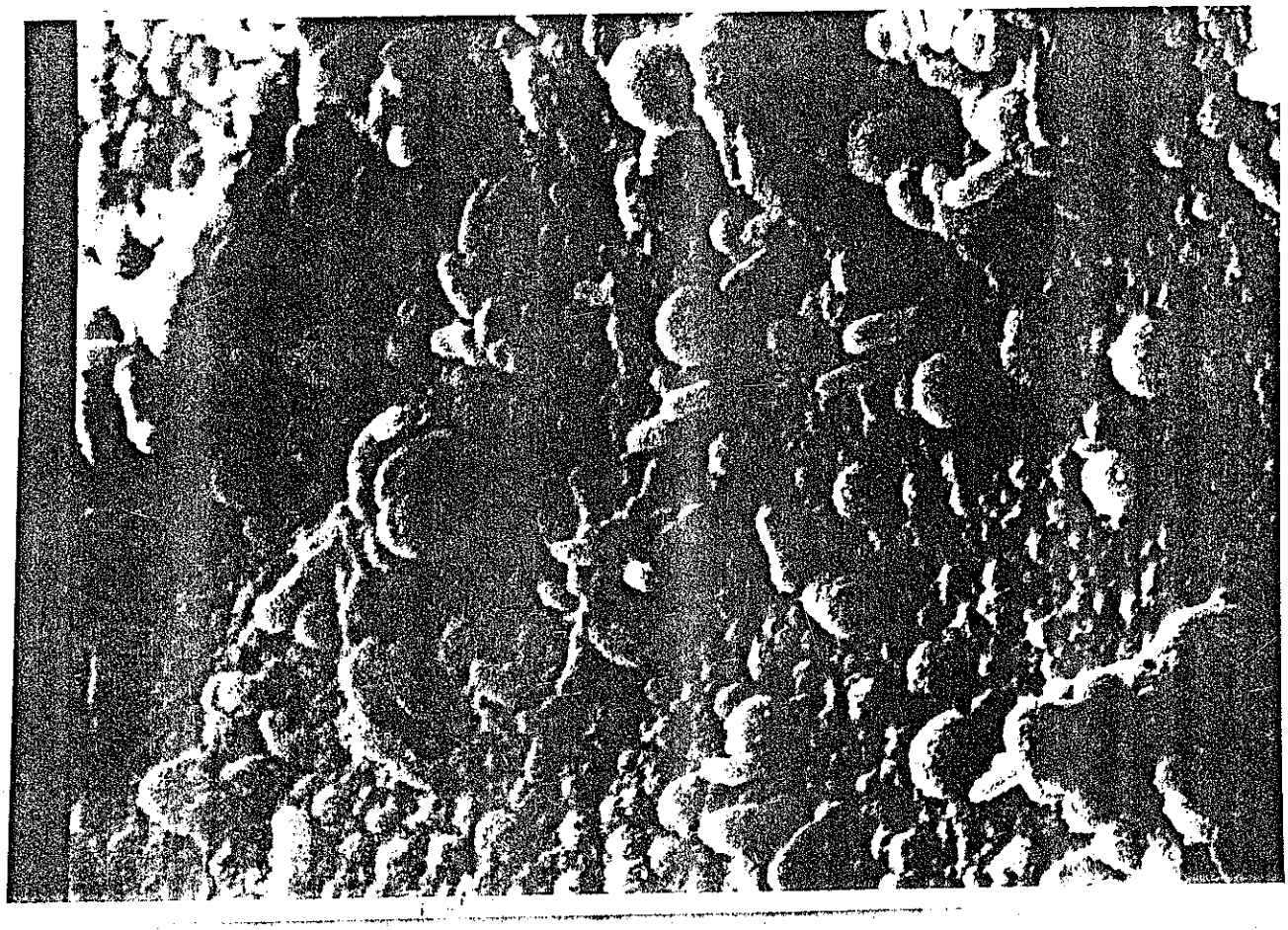
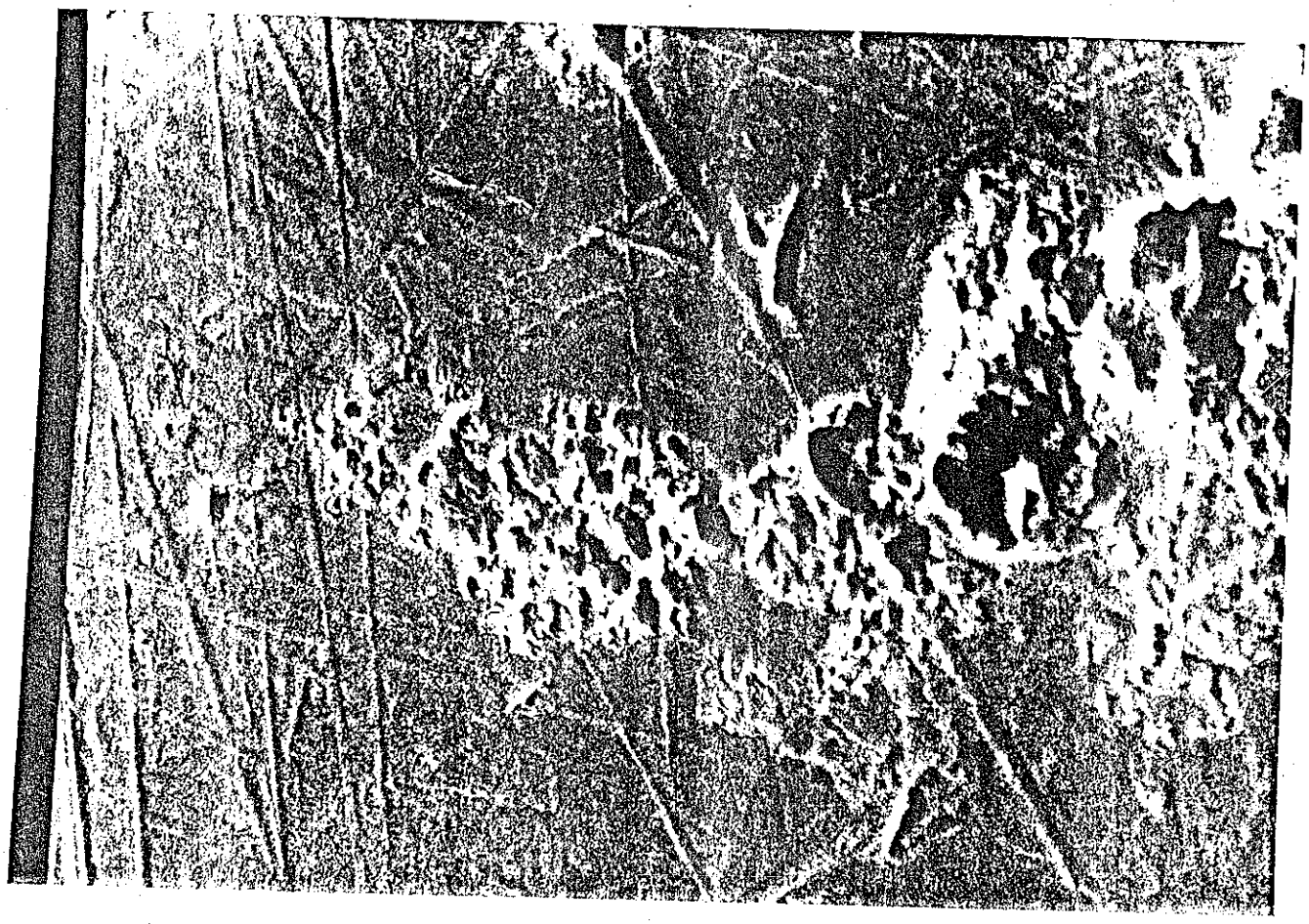


Figure 9. Scanning electron micrograph of the surface of mild steel coupon after removal of the corrosion products shown in Figure 7. Magnification is 1200X.



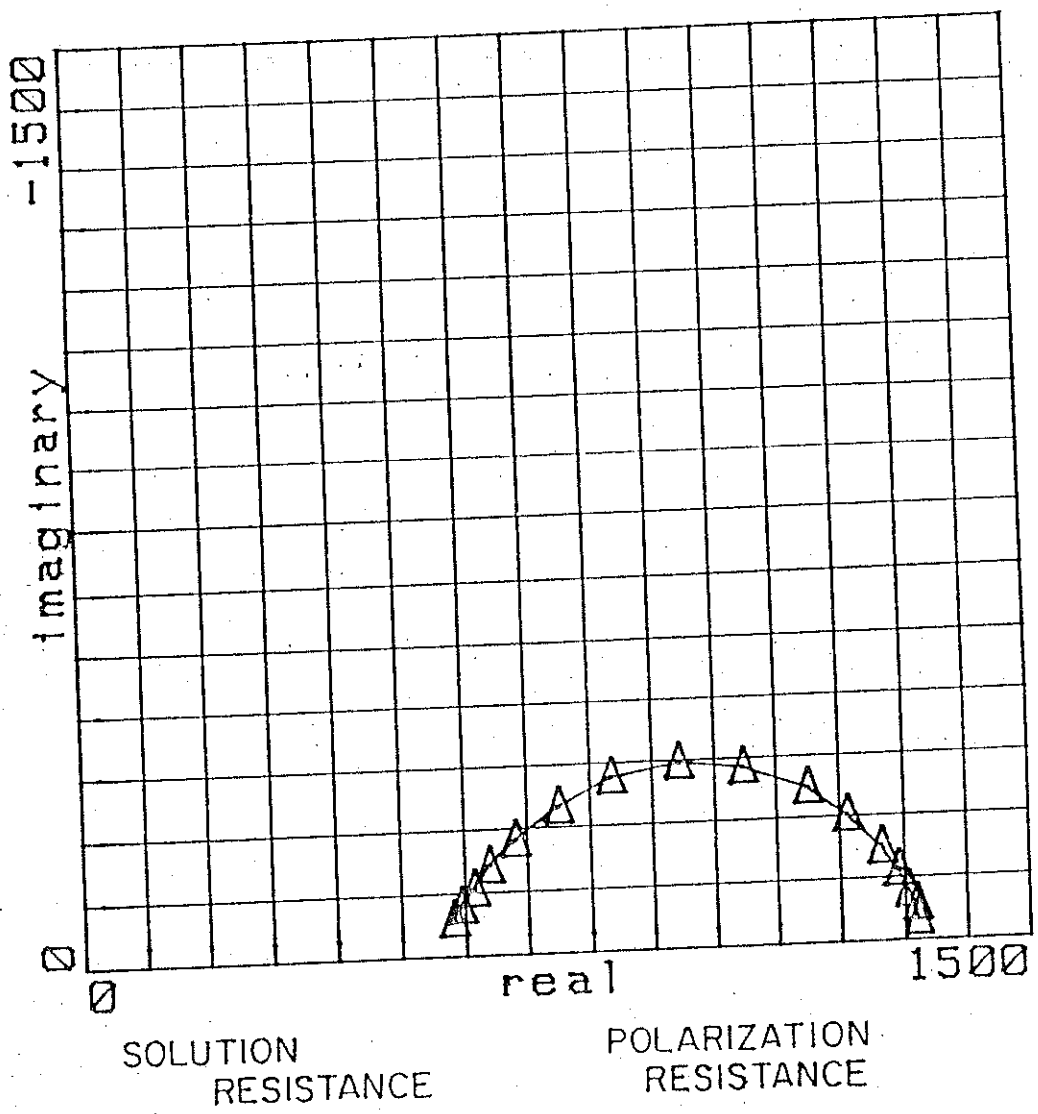


Figure 10. Nyquist complex plane plot of the corrosion on mild steel coupons induced by a biofilm of six physiological groups of MIC related bacteria measured by electrochemical impedance spectroscopy (EIS). The solution resistance can be estimated from the real impedance data as the distance between the high frequency side of the semicircular projection and the origin. The polarization resistance which is related to I_{corr} can be estimated from the projection of the semicircle onto the real impedance axis.

Table 1.

Recoverability as colony forming units of viable from biofilm on C1020 carbon steel, after sublethal and lethal exposure to hypochlorite.

Bacterial type[a]	Sublethal exposure (2ppm)		
	pre-treatment	post-treatment	recovery[b]
1	$2x10^7$	$4x10^7$	$4x10^7$
2	$2x10^7$	$3x10^7$	$4x10^7$
3	$2x10^7$	$5x10^6$	$8x10^6$
4	$1x10^7$	$5x10^7$	$4x10^7$
5	$1x10^7$	$1x10^7$	$2x10^7$
6	$5x10^4$	$1x10^4$	$1x10^4$
	Lethal exposure (16ppm)		
	pre-treatment	post-treatment	recovery
1	$2x10^7$	$6x10^4$	$6x10^6$
2	$2x10^6$	$3x10^3$	$2x10^5$
3	$1x10^7$	$2x10^3$	$5x10^5$
4	$7x10^6$	$1x10^3$	$8x10^6$
5	$2x10^6$	$9x10^3$	$4x10^6$
6	$1x10^6$	$1x10^3$	$7x10^5$

[a]Bacterial types.
1. gram positive, spore forming Bacillus.
2. Iron reducing bacterium
3. Iron precipitating, slime forming, strict aerobe.
4. Acetic acid producing, facultative anaerobe, fast grower.
5. Acetic acid producing, facultative anaerobe.
6. Sulfate reducing bacterium, lactate utilizing.

[b]Recovery denotes 24hrs of no biocide treatment, after the initial treatment.

Table 2.

hospholipid ester-linked fatty acids recovered from corroded coupons, after exposure to sublethal HOCl (and recovery) compared to control.

	Mole %	
PLFAME	Control	Sublethal HOCL
14:0	nd	0.14
iso 15:0	0.30	0.54
a 15:0	nd	0.13
15:0	0.15	nd
iso 16:0	0.20	0.04
16:1w7c	14.31	15.02
16:1w7t	1.41	1.58
16:1w5	0.15	0.27
16:0	24.11	25.75
i17:1	0.65	0.69
iso 17:0	0.14	0.10
a17:0/17:1w8	0.10	0.12
17:1w6	0.05	nd
cy 17:0	6.26	7.50
17:0	0.18	0.26
unidentified	0.58	0.63
18:3w6	0.32	nd
iso 18:0	0.08	0.13
18:1w7c	44.10	40.48
18:1w7t	2.78	3.05
18:0	1.01	1.00
Br19:1	1.55	1.13
cy19:0	0.98	0.98
unidentified	0.59	0.49
Total	100.00	100.00
nmol/coupon	27.60	28.00