



Bacterial colonization and modification of grain boundaries on 316L stainless steel
by Richard John Gillis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Microbiology
Montana State University
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Abstract:

Localized pitting corrosion has become a significant problem with 316L stainless steel used as a piping material for service water systems in nuclear power facilities. Biofilms and the activities of the microorganisms associated with them are frequently implicated. Although much is known about abiotic corrosion, comparatively little is known about microbially influenced corrosion of stainless steel. Current research focused on using surface sensitive techniques including scanning confocal laser microscopy (SCLM), image analysis, energy dispersive X-ray spectroscopy (EDX), Auger electron spectroscopy (AES), and atomic force microscopy (AFM).

A dual species biofilm containing *Pseudomonas aeruginosa* and *Desulfovibrio gigas* was grown on 316L hand polished stainless steel under aerobic conditions. Microcolonies of *D. gigas* were detected using an SRB polyclonal antibody, however stainless steel surface preparation changes discontinued these experiments. A dual species biofilm containing a facultative anaerobic bacterium, *Citrobacter freundii*, and a sulfate reducing bacterium, *Desulfovibrio gigas*, was grown on a 316L 2B finish stainless steel under anaerobic conditions. After 16 days, direct enumeration revealed densities of *C. freundii* on the surface to be approximately $1 \times 10^7/\text{cm}^2$, whereas *Desulfovibrio gigas* was found to be in lower average densities and growing in areas containing *C. freundii*. Using SCLM and image analysis techniques, *C. freundii* was shown to preferentially colonize grain boundaries on the stainless steel surface. AFM showed the grain boundaries were deep and wide enough to harbor bacteria. Subsequent analysis by small spot AES showed a depletion in chromium relative to nickel in the grain boundaries of the coupons exposed to *C. freundii* with or without *D. gigas* and a depletion in iron relative to nickel in the coupons exposed to both *C. freundii* and *D. gigas*. AES also revealed sulfur and phosphorus nutrient deposition within the grain boundaries and may provide rationale for preferential bacterial colonization. The present study demonstrates that bacterial colonization of grain boundaries on 316L SS results in depletion of Cr and Fe relative to Ni. Chromium depletion is thought to promote localized attack on this alloy. Selective removal of Cr and Fe may represent a mechanism of localized microbially influenced corrosion of 316L stainless steel.

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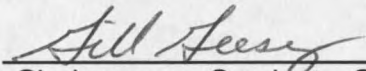
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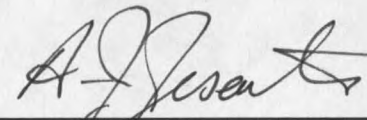
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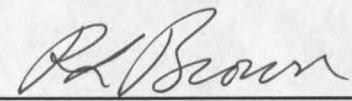
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TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	xii
INTRODUCTION	1
Stainless Steel	1
Stainless Steel Microstructure	2
Surface Analysis of Corrosion	3
Microbially Influenced Corrosion	4
Mechanisms of MIC	5
Cathodic Depolarization	5
Biofilms and MIC	6
Localized Pitting Corrosion	8
Chloride Effects and Stress Corrosion Cracking	8
Hydrogen Embrittlement	9
MIC and Weldments	10
Bacterial Coexistence and MIC	11
Undefined Consortia to Study MIC	11
Defined Consortia to Study MIC	12
Other Potentially Corrosive Bacteria	14
Bacterial Colonization of Stainless Steel	15
Significance of Initial Colonization Events	17
Summary	18
Hypothesis	19
STATEMENT OF RESEARCH PROBLEM	20
MATERIALS AND METHODS	21
Chemicals	21
Equipment and Supplies	21
Reactor Design	23
Coupon Selection and Surface Preparation	24

TABLE OF CONTENTS (Continued)

	Page
Assembly of the Reactor System	25
Bacteria	26
Suspended Cell Growth Studies	27
Culture Medium	29
Enumeration of Suspended Bacteria via Membrane Filtration	29
Studies of Bacterial Colonization of Stainless Steel	
in the Aerobic Reactor	30
Evaluation of Fluorescent Antibodies Used to Detect SRB	32
Fluorescent Antibody Staining to	
Determine Surface-Associated SRB	33
Studies of Bacterial Colonization of Stainless Steel	
in the Anaerobic Reactor	34
Culture Medium	34
Inoculation of Reactor with Mono and Mixed Populations	35
Coupon Sampling	36
Enumeration of Biofilm Bacterial Populations	37
Bacterial Colonization Pattern Analysis	37
Scanning Confocal Laser Microscopy	37
Image Processing and Analysis	38
Energy Dispersive X-ray Spectroscopy (EDX)	40
Auger Electron Spectroscopy	41
Atomic Force Microscopy	43
Statistical Analyses	44
RESULTS	45
Fluorescent Antibodies	45
Significance of Passivation	45
Growth of <i>D. gigas</i> in the Presence and Absence of <i>C. freundii</i>	48
Bacterial Colonization Patterns	54
Surface Analysis	59
Energy Dispersive X-ray Spectroscopy (EDX)	59
Scanning Auger Electron Spectroscopy (AES)	61
Analysis of Non-Alloy Elements	74
Atomic Force Microscopy	76
DISCUSSION	80
Bacterial Colonization of Grain Boundaries	80
Implications of Bacterial Attachment to Grain Boundaries	81

TABLE OF CONTENTS (Continued)

	Page
Bacterial Modification of Grain Boundaries	84
Potential Prevention of MIC on Stainless Steel	88
Conclusions	89
LITERATURE CITED	90
APPENDIX	98

LIST OF TABLES

Table	Page
1. Grid areas at various magnifications	30
2. Combinations of bacteria used for antibody studies	31
3. Combinations of bacteria used for anaerobic experiments	35
4. Average bulk elemental percentages of 316L SS as determined by EDX	41
5. Kinetic energies and sensitivity factors used for elemental percentage calculations	42
6. SRB antibody binding efficiency to <i>D. gigas</i>	45
7. Elemental percentages of chromium, nickel, and iron in 316L stainless steel. Bulk grain (BG) and bulk grain boundary (BGB) measured by EDX, surface grain (SG) and surface grain boundary (SGB) measured by AES, literature values reported as nominal compositions in percentages	59

LIST OF FIGURES

Figure	Page
1. Diagram of reactor used for biofilm studies	24
2. Image showing <i>D. gigas</i> (large vibroid bacteria) and <i>C. freundii</i> (very small rods) stained with DAPI on the same membrane filter at 400X magnification	28
3. Section analysis generated by atomic force microscopy of the 316L stainless steel surface. Arrow color in upper left depth profile corresponds to vertical distance data in lower right	44
4. Biofilm containing <i>C. freundii</i> and <i>D. gigas</i> grown aerobically on hand polished 316L SS. Two illuminations of same field (a) SRB stained with antibody (b) Total cells stained with DAPI. Magnification 1000X	46
5. Epifluorescent micrographs of unstained as-received 316L SS surface, comparing (a) non-passivated to (b) passivated. Autofluorescing material is very abundant in (a). Magnification 1000X	47
6. Bacteria attached to passivated and hand polished SS coupons suspended for 5 days in anaerobic batch cultures, 1 containing <i>D. gigas</i> , 1 containing <i>D. gigas</i> + <i>C. freundii</i> enumerated via DAPI DC	49
7. Anaerobic growth curve of <i>D. gigas</i> (SRB) in the presence and absence of <i>C. freundii</i> (C.f.) via DAPI direct count. Each line represents one batch culture flask at 22°C	50
8. Viable (VC) and direct count (DC) of anaerobic reactor effluent. Line 2 contains only <i>D. gigas</i> (SRB), line 3 contains <i>D. gigas</i> (SRB) + <i>C. freundii</i> (Cf), line 4 contains only C.f.	51
9. Direct counts of SRB on coupons and on total surface area of reactor. SCF (<i>C. freundii</i> + <i>D. gigas</i>). Results represent data from 2 experiments	52

LIST OF FIGURES (Continued)

Figure	Page
10. Epifluorescent micrograph of DAPI stained biofilm of 316L SS from batch culture containing <i>D. gigas</i> and <i>C. freundii</i> . Magnification 1000X	53
11. Total surface associated <i>C. freundii</i> located within grain boundaries (solid bars), compared to total area containing grain boundaries (hatched bars) after 16 days from experiment 1. ND, no data.	55
12. Percentages of total surface-associated <i>C. freundii</i> located at a grain boundary (GB) and percentage of total surface area (grains+GB) contributed by grain boundaries after 16 days in Exp #2. ND, no data.	56
13. Exp #2. Percentage of total surface-associated <i>C. freundii</i> located at a grain boundary (GB) and percentage of total surface area (grains+GB) contributed by grain boundaries after 3 weeks. ND, no data.	57
14. Exp #2. Percentage of total surface-associated <i>C. freundii</i> located at a grain boundary (GB) and percentage of total surface area (grains+GB) contributed by grain boundaries after 4 weeks. ND, no data.	58
15. Conceptual model of stainless steel prior to bacterial colonization showing EDX and AES sampling regions. SG-surface grain, SGB-surface grain boundary, BG-bulk grain, BGB-bulk grain boundary.	60
16. Scanning electron micrograph showing grains and grain boundaries on stainless steel surface. Magnification 2000X.	62
17. Abundance of chromium relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time.	63
18. Abundance of iron relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time.	64

LIST OF FIGURES (Continued)

Figure	Page
19. Abundance of nickel relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time.	65
20. Abundance of chromium relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> + <i>D. gigas</i> after 2, 3, or 4 weeks based on small spot AES after 60 s sputter time.	66
21. Abundance of iron relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> + <i>D. gigas</i> after 2, 3, or 4 weeks based on small spot AES after 60 s sputter time	67
22. Abundance of nickel relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> + <i>D. gigas</i> after 2, 3, or 4 weeks based on small spot AES after 60 s sputter time	68
23. Ratios of chromium to nickel in grains and grain b. of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> with or without <i>D. gigas</i> as determined by small spot AES after 60 s sputter time	69
24. Ratios of chromium to nickel in grains and grain b. of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> + <i>D. gigas</i> for 2, 3, or 4 weeks as determined by small spot AES after 60 s sputter	70
25. Ratios of iron to nickel in grains and grain b. of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> with or without <i>D. gigas</i> as determined by small spot AES after 60 s sputter time	72
26. Ratios of iron to nickel in grains and grain b. of SS coupons exposed to sterile culture medium (Con) or to <i>C. freundii</i> + <i>D. gigas</i> for 2, 3, or 4 weeks as determined by small spot AES after 60 s sputter	73

LIST OF FIGURES (Continued)

Figure	Page
27. Abundance of nitrogen relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time	75
28. Abundance of sulfur relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time	77
29. Abundance of phosphorus relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to <i>C. freundii</i> (Cf) with or without <i>D. gigas</i> (SRB) based on small spot AES after 60 sec sputter time	78
30. Atomic force micrograph of 316L stainless steel surface showing three-dimensional image of surface	79
31. Conceptual model of initial corrosion events, whereby a patchy mixed culture biofilm is covering a grain boundary and causing the selective removal of chromium (c) and iron (f)	87

ABSTRACT

Localized pitting corrosion has become a significant problem with 316L stainless steel used as a piping material for service water systems in nuclear power facilities. Biofilms and the activities of the microorganisms associated with them are frequently implicated. Although much is known about abiotic corrosion, comparatively little is known about microbially influenced corrosion of stainless steel. Current research focused on using surface sensitive techniques including scanning confocal laser microscopy (SCLM), image analysis, energy dispersive X-ray spectroscopy (EDX), Auger electron spectroscopy (AES), and atomic force microscopy (AFM).

A dual species biofilm containing *Pseudomonas aeruginosa* and *Desulfovibrio gigas* was grown on 316L hand polished stainless steel under aerobic conditions. Microcolonies of *D. gigas* were detected using an SRB polyclonal antibody, however stainless steel surface preparation changes discontinued these experiments. A dual species biofilm containing a facultative anaerobic bacterium, *Citrobacter freundii*, and a sulfate reducing bacterium, *Desulfovibrio gigas*, was grown on a 316L 2B finish stainless steel under anaerobic conditions. After 16 days, direct enumeration revealed densities of *C. freundii* on the surface to be approximately $1 \times 10^7/\text{cm}^2$, whereas *Desulfovibrio gigas* was found to be in lower average densities and growing in areas containing *C. freundii*. Using SCLM and image analysis techniques, *C. freundii* was shown to preferentially colonize grain boundaries on the stainless steel surface. AFM showed the grain boundaries were deep and wide enough to harbor bacteria. Subsequent analysis by small spot AES showed a depletion in chromium relative to nickel in the grain boundaries of the coupons exposed to *C. freundii* with or without *D. gigas* and a depletion in iron relative to nickel in the coupons exposed to both *C. freundii* and *D. gigas*. AES also revealed sulfur and phosphorus nutrient deposition within the grain boundaries and may provide rationale for preferential bacterial colonization. The present study demonstrates that bacterial colonization of grain boundaries on 316L SS results in depletion of Cr and Fe relative to Ni. Chromium depletion is thought to promote localized attack on this alloy. Selective removal of Cr and Fe may represent a mechanism of localized microbially influenced corrosion of 316L stainless steel.

INTRODUCTION

Stainless Steel

Stainless steel has been used as a piping material for many years and its metallurgical properties are well characterized (80). A variety of different types of stainless steel alloys are available, however austenitic stainless steels are most commonly used for piping materials. Austenitic stainless steels, by definition, are of face-centered cubic lattice. This allows them to have high ductility and tensile strength making them ideal candidates for tubing and piping fabrication. Austenitic steels consist of the American Iron and Steel Institute (AISI) 300 series stainless steels, most commonly 304, 304L, 316, and 316L. These stainless steels contain nickel, iron, chromium, molybdenum, manganese, silicon, and less than 1% carbon, phosphorus, and sulfur depending on the particular alloy. These particular stainless steels are designed to have increased corrosion resistance due to their alloy composition (43). The high chromium content (as high as 20%) reduces corrosion by forming a passive film of chromium oxide across the surface (58). Low carbon content reduces intergranular corrosion caused by the migration of carbides to the grain boundaries. Molybdenum enhances the corrosion resistance to pitting and crevice corrosion by allowing formation of a more uniform passive layer (47). Interestingly, Scott, *et al*, report that there may be some type of maximum concentration of molybdenum that enhances corrosion resistance and

that 316L may contain the optimal concentration for resistance to microbially influenced corrosion (MIC) (61). Because molybdenum is a potent ferritizer, nickel must be present in fairly high concentrations (10%) to prevent delta ferrite formation, which may be structurally detrimental to the stainless steel, as will be discussed later. A passive chromium oxide layer forms on the surface of these alloys under aerobic conditions. This passive layer forms an electronic insulating barrier decreasing the electric field strength and cationic transport into solution that occurs during the corrosion process (22). If this oxide layer is breached, either biotically by the activity of microorganisms or abiotically by welding and heating, corrosion can occur at an accelerated rate (69).

Stainless Steel Microstructure

The fabrication of stainless steel causes it to be metallurgically separated into areas of grains and grain boundaries. During the annealing process, cations nucleate crystal growth. As adjacent crystals (grains) converge, boundaries are formed between them, termed grain boundaries. These grain boundaries tend to be higher in carbon and other impurities due to carbide precipitation (6,8,39) which causes localized breakdown in the oxide film (1,69) and have a thinner chromium oxide surface layer as well as a lower general amount of chromium (up to 40% less) than the adjacent grains in instances where extensive carbide precipitation occurs (7). The metallurgy of the grains and grain boundaries in stainless steel has been extensively studied using a multitude of surface analytical instruments (ie. scanning Auger spectroscopy, energy dispersive x-ray spectroscopy, x-ray

photoelectron spectroscopy, scanning tunneling microscopy, transmission and scanning electron microscopy, etc.) (10,23,36,44,47,49,50,58). Most of the current research has analyzed metallurgical stresses that stainless steel undergoes in certain situations, such as high temperatures and pressures, and how grain boundary composition and structure are affected. Many studies have been published using the aforementioned surface techniques to investigate corrosion of stainless steel. McIntyre (1983) reviewed the most frequently used instruments, Auger electron and X-ray photoelectron spectroscopies, to study corrosion. Quantification still seems to be a challenge for these analytical techniques (47).

Surface Analysis of Corrosion

More recently, new applications have been reported using other types of surface analytical techniques. Fan and Bard (1989) (23) investigated corrosion of 304L stainless steel in aqueous chloride media using scanning tunneling microscopy, however results reported were more of a qualitative than quantitative nature. The majority of the past studies have focussed on abiotic corrosion, however some scientists have used these surface analytical instruments to investigate MIC. Chen, *et al* (1988) (12) used scanning Auger electron spectroscopy to investigate biological corrosion of copper, titanium, aluminum brass, and stainless steel suspended in the open ocean for 56 and 92 days. Unfortunately, no chemical characterization of the stainless steel surface was reported, other than to note that the surface "appeared" visually corroded. Because of the difficulties of combining microbiological and surface chemical

analysis, not much research has been done in this area (Gustavo Cragnolino, Jeff Kearnes, personal communication).

MIC of stainless steel has been studied on a larger scale using electrochemical techniques. Mansfield, *et al*, (1992) (46) studied 304 and 316 stainless steels exposed to flowing seawater. After 124 days they reported no corrosion either visually, microscopically, or electrochemically, measured by open circuit potential. Studies are ongoing in this area, but laboratory study of microbially influenced corrosion has proved challenging and difficult to reproduce under defined conditions therefore warranting further investigation.

Microbially Influenced Corrosion

The corrosion process from an abiotic standpoint has been well characterized but, in many ways, the processes of MIC remain elusive and somewhat speculative (27,33,77). A growing appreciation of the significance of MIC in corrosion processes has led to a resurgence in investigation. Recent studies have primarily focussed on mild steel (17,18,32,34,48) due to its extensive use in industrial water systems. Stainless steels were generally thought to be corrosion "resistant" and thus were the material of choice in applications where corrosion resistant materials were needed (33). These situations include power generating industries, chemical plants, paper mills, waste water systems, etc (31,79). Localized corrosion of stainless steel piping, usually in the form of pitting, has been documented, especially in areas of stagnant or low to intermittent flow (31,37,42,68,78,79). It has been particularly troublesome in the nuclear power

industry where service water systems may be filled with untreated water and left stagnant for months prior to placing into service, resulting in rapid extensive corrosion and subsequent system failure (78). Some temporary remedies included welding new stainless steel sleeves over corroding stainless steel pipes, however this is usually temporary, with system failure occurring as soon as 2 years (68). Once MIC occurs, it is difficult to halt and until a better understanding of the mechanisms and means of initiation are known, the problem will continue.

Mechanisms of MIC

Cathodic Depolarization

MIC is a phenomenon that was hypothesized by von Wolzogen Kühr and van der Vlugt in 1934 (74). They speculated that anaerobic corrosion of buried iron pipes was caused by the presence of a sulfate reducing bacterium, *Desulfovibrio desulfuricans*. Based on the research of Stephenson and Strickland (66), who had shown that this bacterium was able to use molecular hydrogen for the reduction of sulfate to hydrogen sulfide, von Wolzogen Kühr and van der Vlugt proposed the theory of cathodic depolarization, being applicable to all materials containing iron. In this theory, the iron surface is negatively polarized in water by losing metal cations (anodic reaction). The electrons left on the surface reduce protons from the water to atomic hydrogen (cathodic reaction). The bacterial hydrogenase scavenges the atomic hydrogen to reduce sulfate, thus acting as a depolarizing agent (cathodic depolarization reaction). This action eliminates the build up of hydrogen on the surface, which is thought to polarize the cathodic

reaction, although hydrogen accumulation may cause other problems such as stress corrosion cracking (26). From the inception of cathodic depolarization, researchers have studied MIC and appended the theory as more became known (32,57). A number of reviews (14,26,27,33,60,73) have been written on the subject as the theory is updated, but much remains unknown and speculative. Consequently, other localized corrosion mechanisms have also been proposed.

Biofilms and MIC

The idea of biofilm formation on a surface and its association with MIC is a relatively new idea when compared to the previously discussed cathodic depolarization theory. Nonetheless, this area of study has spawned a number of new theories about MIC (73). On stainless steel, breaching of the passive layer, generally considered to be chromium oxide is one means by which biofilms can contribute to MIC (27). As previously mentioned, the chromium oxide layer is one of the major characteristics of stainless steel that enhances corrosion resistance (43). Biofilm detachment, which may occur more frequently under differential flow conditions (73), may expose a breached passive layer, whereby the protective chromium oxide layer is no longer intact. This breaching may be caused by the formation of crevices, differential aeration zones, or a more aggressive environment beneath the biofilm (31). Once this passive layer is breached, corrosion can occur at an accelerated rate if no repassivation occurs as might be the case under anaerobic conditions.

With the chromium oxide layer being more unstable at the grain boundaries,

a corrosive biofilm may be able to breach the passive oxide layer in this area more rapidly than at the grains. Once the oxide layer is breached, bacteria can then assist in dissolution of the metal (31). Bacteria may contribute to certain anodic effects by production of organic acids such as short chain fatty acids, thereby increasing the proton concentration (17,27,33). The production of sulfides such as FeS and H₂S by SRB may have an anodic or cathodic effect depending on the specific conditions of that particular surface area. SRB are generally considered to be the major culprits in MIC of stainless steel. It has been reported that SRB are responsible for over 77% of corrosion that occurred in a specific oilfield (78). Others have speculated that annual monetary loss due to MIC caused by SRB is in the billions of dollars per year (27,33). Consequently, most laboratory studies on MIC have used SRB to mimic corrosive field conditions.

Patchy biofilms containing aerobic organisms may also accelerate corrosion by creating concentration cells along the substratum. Exopolymeric substances (EPS) produced by many attached bacteria may have this effect (4,53). The EPS can contain certain acids (ie uronic acids) that may have a corrosive nature. Costerton and Geesey (14) have proposed the formation of a corrosion cell where there are localized anodic and cathodic sites due to microbial activity on the surface. This phenomenon may be due to aerobic bacteria producing EPS, but it may also be due to SRB as well as iron-oxidizing bacteria. If the SRB are metabolically active, they will be producing sulfides which have been shown to be very corrosive (33). Iron-oxidizing bacteria such as *Gallionella* have been

implicated in corrosion of stainless steel (42). These bacteria have the ability to produce sulfuric acid, which is very corrosive to stainless steel (33).

Localized Pitting Corrosion

The most common type of MIC to occur on stainless steel is that of localized pitting corrosion associated with biofilms (22,31,33,61). Pits generally begin as small "pinholes" that can expand into large cavities within the bulk metal. Pit formation is thought to be caused by a hydrolysis reaction which both concentrates chlorides and acidifies the immediate environment within the pit (22). Microbial activity, particularly of SRB, is thought to propagate the phenomenon (27). The formation of oxygen concentration cells caused by the presence of biofilms, as aforementioned, can also cause pit enhancement (22). Microbial activity can also be responsible for initial pit formation by causing the adsorption of aggressive ionic species, as discussed below.

Chloride Effects and Stress Corrosion Cracking

Once a biofilm has been established on a stainless steel substratum, it has the ability to trap and entrain certain chemical species that may be particularly aggressive toward stainless steel (73). Chloride is one such chemical that has been shown to be of particular importance (43,60). Chloride concentrations have been found to be surprisingly high within an established tubercle or corrosion deposit, even when the chloride concentration in the surrounding medium is quite low (5,68). Certain species of iron-oxidizing bacteria, ie *Gallionella*, have the ability

to concentrate chlorides, usually complexed as ferric and manganic chlorides within the tubercle (70). Chloride ions have the ability to permeate the passive film thus initiating or hastening the electrochemical reactions that are responsible for corrosion. The effect can be two-fold. Chloride can cause small pits to form beneath the tubercle that can penetrate stainless steel piping walls in as short a time as a few weeks (37). In addition, chloride initiates the phenomenon known as stress corrosion cracking that affects all alloys and has been well characterized in stainless steel (43). Stress corrosion cracking begins at the anodic site and causes metal dissolution to occur at the crack front. A brittle fracture results which is transgranular in austenitic stainless steels. Once this fracture has occurred, it will enlarge to the point of material failure.

Hydrogen Embrittlement

Hydrogen embrittlement is another means by which MIC can cause material failure of stainless steel (25). Hydrogen embrittlement involves atomic hydrogen entering the stainless steel lattice causing loss of ductility, tensile strength, and crack propagation. Any bacterium that produces an acid byproduct may be a problem, as well as SRB which produce sulfides that prevent transformation of atomic hydrogen to molecular hydrogen. Some SRB can consume hydrogen, thus cathodically depolarizing the stainless steel. The overall outcome of hydrogen embrittlement may be determined by the competition between the metal, hydrogen producing bacteria, and hydrogen consuming bacteria. Since the metal surface characteristics will determine the number of adsorption sites for the atomic

hydrogen, biofilm composition remains the controlling variable for hydrogen adsorption.

The microstructure of the stainless steel surface, primarily the grain boundaries, could be ideal regions for hydrogen embrittlement. As was aforementioned, these areas are inherent "weak spots" on the stainless steel surface. If there was preferential bacterial attachment to these areas, hydrogen embrittlement could occur.

MIC and Weldments

For reasons not entirely understood, MIC is more frequently reported at or near a weld than any other area on a stainless steel surface (5,31,44). Welding of stainless steel leads to a biphasic condition, where some of the austenite (face-centered cubic) is converted to delta ferrite (body-centered cubic). The delta ferrite phase is generally more susceptible to corrosion than the austenitic phase, however, the austenitic matrix may be preferentially attacked under oxidizing acidic conditions (35). Reducing, acidic conditions tend to favor attack at the delta ferrite regions (31). The biphasic condition seems to be more susceptible to MIC. This condition is known to occur in heat affected zones (HAZ) and has been shown to be susceptible to MIC (31,42). This may be due to the fact that the HAZ is sensitized during welding, causing chromium carbide precipitation at the austenite-ferrite interphase. As a result, chromium depleted zones are created (7) which will be subject to MIC (31). The weld metal is also more susceptible to MIC in comparison to the base metal (5,31). Although a variety of different weld metals

have been tried, so far all have shown susceptibility to MIC. Little investigation has been done into why this may occur (5).

Bacterial Coexistence and MIC

MIC of metals is frequently reported to occur to greater extent in aerobic systems than anaerobic systems (33). As aforementioned, SRB are considered to be the most problematic in association with MIC and are commonly isolated from corrosive biofilms (27,47). Because SRB require anoxic conditions for growth, many reviews on MIC have speculated that biofilms provide these conditions even in systems where the water contains oxygen (14,27,73). Hamilton (1985) reports highest sulfate reduction rates occur near the aerobic/anaerobic interface (27). This implies that the occurrence of SRB in aerobic environments may not be improbable, provided there are small anaerobic microzones for sulfate reduction to occur. Microorganisms undergoing aerobic metabolic processes could create such zones (27,73). Other metabolic "cross-feeding" may also be involved. For SRB to be growing in these "microenvironments", an intricate symbiosis has evolved. In an attempt to study these phenomena, researchers have tried to recreate conditions that may initiate MIC.

Undefined Consortia to Study MIC

One approach to studying MIC has been to use undefined consortia to gain information about how these events may occur. Scotto, *et al.*, (1985) investigated the corrosive behavior of an undefined marine aerobic biofilm on 304 and 316

stainless steel (63). Although their results were qualitative, they did report the "microbiota" had corroded the stainless steel, evidenced by crevice corrosion on loops fed with natural seawater. They speculated that this was due to the breakdown of the passive film followed by nucleation of localized attack, although they provided no support for these findings. Zambon, *et al.*, (1984) investigated marine fouling films on mild steel. They used specific polyclonal antibodies to identify various microbial species they suspected were growing in the films produced from an undefined sea water inoculum. Unfortunately, because an undefined seawater inoculum was used to grow the films, it was impossible to prove that there was no cross reactivity of the antibodies. A multitude of studies have been carried out using undefined consortia (27,73), but traditionally, results are virtually impossible to reproduce. It is for this reason that others have attempted to reproduce corrosion using defined systems.

Defined Consortia to Study MIC

Many researchers have attempted to study MIC by using organisms reported to cause MIC, primarily SRB, in an attempt to mimic corrosive biofilms. Jack (1990) constructed a three member bacterial consortium consisting of an obligate aerobe, a facultative anaerobe, and an SRB (34). The corrosion rate of mild steel, measured by open circuit potential, was reported to be highest in the presence of the SRB. Dowling, *et al.*, (1992) created a consortium of a *Eubacterium* sp. and two sulfate reducing bacteria on a carbon steel surface. They used scanning electron microscopy coupled with electrochemical impedance

spectroscopy to measure the corrosion rate. They speculated that both the fermentative and sulfate reducing bacteria caused an increase in the corrosion rate. Beech, *et al.*, (1991) measured the amount of exopolysaccharide (EPS) produced by *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* in the presence of mild and stainless steel (British Standard 02134) (4). They reported that the amount of biofilm present on the stainless steel was much less than that on the mild steel and suggested that some EPS may be aggressive to metal dissolution due to high acid concentration at the surface. Dowling, *et al.*, (1988) used a mixed culture of *Vibrio natriegens*, *Desulfovibrio* sp., and *Desulfobacter* sp. to investigate corrosion of mild steel (18). Measuring corrosion via electrochemical impedance, they reported highest corrosion rates when both SRB and *Vibrio natriegens* were present. Other researchers have also shown defined mixed cultures containing SRB to be corrosive to stainless steel. Deshmukh, *et al.*, (1992) showed that cultures of *Comamonas testosteronii* and *Desulfovibrio vulgaris* caused more extensive localized pitting corrosion, as measured by pit size and number, on 304 stainless steel than either culture alone (16). Research and field observation have given some clues as to what types of bacteria may cause or initiate MIC. Certainly in natural environments, more than one or two organisms work together to carry out this process. As a result, other types of bacteria have been implicated as well.

Other Potentially Corrosive Bacteria

Numerous researchers have proposed other bacteria that may also be responsible for MIC of various iron and iron-based alloys, including stainless steel. In 1949, Olsen and Szybalski (55) reported metal oxidizing bacteria that could accelerate the corrosion of cast iron. Iron oxidizing bacteria, i.e. *Gallionella sp.* have been reported to cause corrosion in a variety of industrial systems (19,27,33,42,73). Iron oxidizing bacteria produce sulfuric acid, which is very corrosive to most metals (42). Others have shown additional types of bacteria may also be responsible. Nivens, *et al.*, (1986) reported a 13 fold increase in corrosion current density on 304 stainless steel using the marine bacterium *Vibrio natriegens* grown in seawater (53). Obuekwe, *et al.*, (1986) concluded that energy was a limiting factor in the corrosion of mild steel by a *Pseudomonas sp.* (54). Others have speculated that bacteria capable of growing facultatively could produce organic acids that could be corrosive in a localized manner (27,73). Manganese-oxidizing bacteria have also been reported in causing MIC of 304 stainless steel in a service water system at a nuclear power facility (68). A multitude of metabolically different organisms have been implicated in causing or initiating MIC. For bacteria to cause MIC, they must first be attached to the substratum. As prevention of MIC is easier than control of these corrosion processes, other researchers have investigated bacterial colonization in hopes of discouraging attachment.

Bacterial Colonization of Stainless Steel

Zobell (81) was one of the first to propose bacterial attachment to surfaces when describing attachment of marine bacteria to sampling bottle walls. He speculated that bacteria were attached due to nutrient availability at the interface. Since that time, a multitude of studies have been undertaken to determine why bacteria attach to surfaces and how attachment may be discouraged in order to prevent bacterial fouling and corrosion of surfaces (21). It is widely accepted that once a bacterium attaches, if conditions are conducive to growth, a biofilm can develop over time. It is also accepted that bacteria can attach to virtually any surface, however attachment to some surfaces may occur much more readily than to others.

Relationships between surface free energies, surface charge, and bacterial attachment have been described to further understanding of the attachment process (2,9,21,24,71,72). Due to its ease in investigation, most work has been done on plastic or glass, however some information is available on stainless steel (21). One of the more defined studies was reported by Vanhaeke, *et al*, (1990) who investigated attachment of *Pseudomonas aeruginosa* to 304 and 316L stainless steel electropolished and 120 grit mechanically polished surfaces in static systems (71). They reported that bacterial cell surface charge (bacterial cell surface hydrophobicity measured by the bacterial adherence to hydrocarbons test and the contact angle measurement test) may be of minor importance in adhesion to stainless steel as bacterial attachment was very similar and independent of the

stainless steel type or surface. Mueller, *et al.* (1992) described initial attachment of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* to hand polished 316 stainless steel, copper, silicon, and glass in a laminar flow system (51). They found that the adsorption rate was positively correlated with surface free energy, surface roughness of the substratum, and the hydrophobicity of the cells.

General "attachment" of specific species of bacteria to stainless steel surfaces has been investigated. Traditionally, little relevance has been given to either the type or surface treatment of stainless steel. Some work has been done for the milk industry using pure cultures of bacteria (28,38,45,64,67,82). Although details were given to the type and culturing of the bacteria used in these studies, little effort was made to define surface features of the substratum before, during, or after bacterial colonization. Bacterial attachment to stainless steel in drinking water distribution systems has also been investigated. Pedersen (1990) (56) examined biofilm development of an undefined mixed culture on a bright annealed or electropolished stainless steel compared to polyvinyl chloride (PVC) pipe in an attempt to compare surface roughness and wettability properties. Although the surface finish of the stainless steel was reported, no mention was made as to the type of stainless steel that was used.

The effects of environmental conditions on bacterial attachment to stainless steel have also been investigated. Stanley (1983) investigated irreversible attachment of *Pseudomonas aeruginosa* to 304 stainless steel in static conditions and reported that growth phase, pH, and motility may influence attachment (64).

Duddridge, *et al*, (1982) examined the effects of surface shear stress on attachment of *Pseudomonas fluorescens* to 316 stainless steel and reported that high shear stress may not prevent microbial attachment (20). Some research has been conducted on bacterial colonization of stainless steel, however surface preparation and characterization are often neglected and no information is currently available on bacterial attachment to stainless steel in relation to stainless steel microstructure.

Significance of Initial Colonization Events

The study of initial colonization events may be integral to understanding MIC of stainless steel. The stainless steel microstructure and initial colonization events by bacteria may be intimately responsible for MIC (44). Primary colonizing bacteria attach at grain boundaries and become metabolically active to form microcolonies. An ideal primary colonizing bacterium would be an organism that can grow well under low nutrient environments and is very "sticky" (52). Many aerobic bacteria and some facultative bacteria, which could be corrosive under the proper conditions and have been shown to readily attach to stainless steel surfaces in low nutrient environments, could be envisioned to fit this scenario (51,52,65). If environmental conditions are conducive to growth, these bacteria will begin to multiply, forming small microcolonies on the surface. Within these microcolonies, microniches form that may be conducive to growth of other types of bacteria, ie sulfate reducing bacteria (SRB). One can envision this consortium becoming a very complex and dynamic ecological setting with small microenvironments

throughout (27). Depending on the type of organisms and the environment, this biofilm could be corrosive to the stainless steel surface, as aforementioned.

Summary

For MIC to occur on stainless steel, bacteria must first attach to the surface. Stainless steel microstructure contributes to bacterial spatial distribution during early biofilm formation as will be presented in this study. Some of these microstructure regions, such as grain boundaries, may be inherent weaknesses in the corrosion barrier of the stainless steel. If bacteria attach to these potentially sensitive areas localized pitting corrosion may occur. As was previously mentioned, abiotic corrosion of stainless steel has been extensively studied, although biotic corrosion of stainless steel in a localized manner has not. In order to more thoroughly understand this problem, further characterization of bacterial attachment to stainless steel and correlation to surface chemistry is needed. Traditionally, little credence has been given to either the surface, surface preparation, or types of bacteria used. The objectives of this study are to use a specific surface, a specific surface preparation, and defined types of bacteria to more clearly define the role of microbes in the corrosion of stainless steel. This study investigates this unique endeavor using a novel combination of surface analytical (Auger electron spectroscopy, energy dispersive X-ray spectroscopy, atomic force microscopy), microscopic (scanning confocal laser microscopy), and computerized image processing techniques to address this problem.

Hypothesis

The hypothesis of this study was that bacterial attachment to stainless steel occurs in a non-random manner and that stainless steel microstructure (grain boundaries) contributes to this process.

STATEMENT OF RESEARCH PROBLEM

The purpose of this research was to conclusively prove whether or not bacteria could selectively attach to grain boundaries on the surface of 316L stainless steel. The second objective was to prove whether or not bacteria could change the surface chemistry of the stainless steel in such a manner that could prove detrimental to material life.

METHODS AND MATERIALS

Chemicals

The following were obtained from Sigma Chemical Co., St. Louis, MO: acridine orange; 4,6 diamidino-2-phenylindole (DAPI); sodium lactate; sodium succinate; sodium sulfate.

The following chemicals were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ: ammonium nitrate; dibasic potassium phosphate; monobasic potassium phosphate.

MacConkey agar and yeast extract were obtained from Difco Laboratories, Detroit, MI.

Ferric chloride and L(+) ascorbic acid were obtained from Matheson, Coleman, and Bell, Norwood, OH. Sodium sulfite was obtained from General Chemical Company, New York, NY.

Resazurin was obtained from Manufacturing Chemists, Norwood, OH.

Equipment and Supplies

The following supplies were obtained from Fisher Laboratory Supplies, Santa Clara, CA: sterile cell scrapers; multi-well cover slides; frosted cover slides; cover slips.

The following supplies were obtained from Gelman Sciences, Ann Arbor, MI:

0.2 μ m pore size Acrodiscs.

The polycarbonate 0.22 μ m filters came from Poretics Corporation, Livermore, CA. The 316L 2B finish stainless steel was obtained from Metal Goods Services, Seattle, WA.

The following supplies were obtained from Cole-Parmer, Niles, IL: silicone tubing. The tubing (Manosil silicone tubing 3/32" ID, 1/32" wall) clamped into the pump cassettes was obtained from Manostat Corp., New York, NY.

The following items of equipment were used in this study: Peristaltic Pump (Manostat model 72-500-000, Manostat Corp., New York, NY); Four peristaltic pump cassettes (Manostat model 72-550-000, Manostat Corp., New York, NY); Ten liter polypropylene carboy (Nalgene, location); Anaerobic chamber (model #12356, Coy Laboratory Products, Ann Arbor, MI); scanning electron microscope (JEOL model JSM 6100, Peabody, MA) equipped with an energy dispersive X-ray spectrometer and software (Noran Instruments, Middleton, WI); scanning Auger electron spectrometer (Perkin-Elmer, Physical Electronics Division, Phi Model 595, Eden Prairie, MN) equipped with a scanning Auger microprobe (Physical Electronics Instruments, Inc., Eden Prairie, MN); membrane filtration towers (Millipore Corp., Cambridge, MA), Gast Vacuum pump (Model#DOA-P104-AA, Gast Manufacturing Corp., Benton Harbor, MI).

The following items of equipment were used for the microscopy: Olympus BH2 microscope (Olympus Optical Co., Ltd., Japan) with an Olympus BH2-RFL-T2 mercury lamp as the light source. An Olympus model UG1 filter (excitation 340

nm, barrier filter 420 nm) was used for viewing DAPI stained preparations, an Olympus model BP490 filter (excitation 490nm, barrier 515nm) was used for viewing acridine orange stained preparations, and an Olympus model BP545 filter (excitation 545 nm, barrier 590 nm) was used for viewing TRITC stained preparations. An Olympus D Plan Apo UV series 160/1.30 oil immersion objective was used for epifluorescent microscopy. Photomicrographs were taken using Kodak Ektar 1000 film (Eastman Kodak Co., Rochester, N.Y.) using a Olympus camera (Model C-35AD-2).

Reactor Design

The reactor system was designed to allow multiple parallel tubing to originate from a common medium container (See Figure 1). Each line of tubing was connected to an experimental chamber containing a stainless steel coupon. Each of three chambers was inoculated with a different matrix of bacteria while the fourth chamber was maintained as a sterile control as will be described. Each chamber contained one 316L 20 gauge 2B finish nitric acid passivated stainless steel coupon (20 cm x 2 cm). The flow rate of medium through the chamber was approximately 20 ml/hour. The flow rate was set to mimic low flow conditions where corrosion problems on stainless steel are generally experienced in industrial systems (Susan Borenstein, personal communication). The volume of the chamber containing the stainless steel coupon was approximately 60 ml, consequently the residence time was approximately 3 hours.

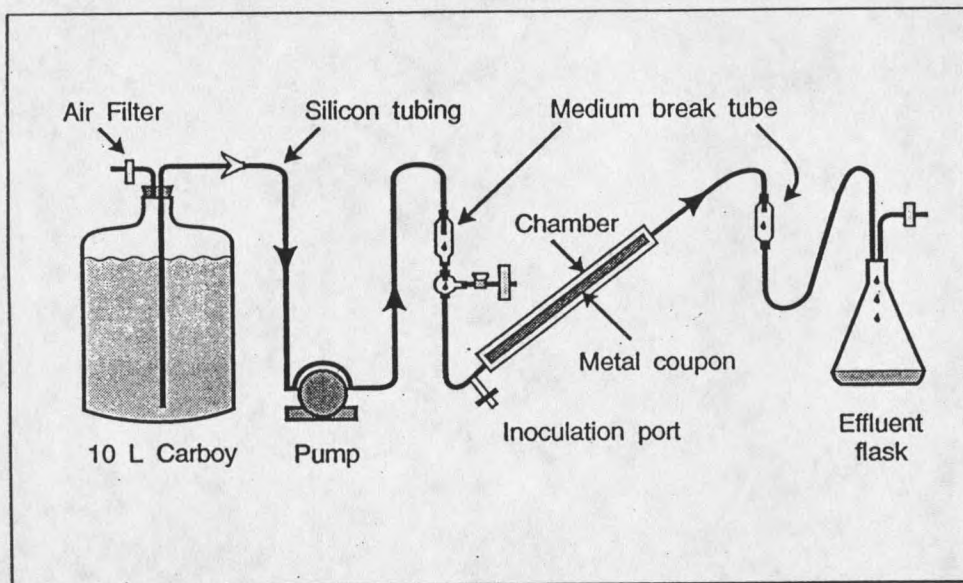


Figure 1. Diagram of reactor system used for biofilm studies.

Coupon Selection and Surface Preparation

A stainless steel material (316L) was selected because of its relevance to the nuclear power industry. This stainless steel is frequently used in the nuclear power industry due to its corrosion resistant properties (Jeff Kearnes, personal communication). The coupon surface was bright annealed classified as 2B finish by AISI. The 2B surface was passivated as is done in most industrial piping

systems using a standard industrial procedure according to the American Society for Testing and Materials procedure #A-380-78 (see #1 in Appendix). This procedure is performed to remove slag and grit which may accumulate on the surface during manufacture and piping installation.

The 316L stainless steel coupons used for the antibody experiments were hand polished using a 0.3 μm grit alumina polish (Buehler, Lake Bluff, IL) for 5 minutes, followed by a 0.05 μm grit alumina polish (Buehler, Lake Bluff, IL) for 5 minutes.

Assembly of the Reactor System

A ten liter polypropylene carboy sealed with a #16 rubber stopper was used to hold the sterile culture medium. Four borosilicate glass tubes were used to draw the medium from the carboy. A stoppered test tube embedded within the rubber stopper was used to aseptically transfer concentrated media into the carboy. All tubing used for the reactor system was either borosilicate glass or silicone. Two medium break tubes were placed between the peristaltic pump and the silicone tube chamber containing the stainless steel coupon to minimize back growth and contamination of the carboy. Another media break tube was located downstream from the chamber to minimize potential contamination from the effluent flask. The entire reactor system was assembled, connected to the carboy containing 9 liters reverse-osmosis (RO)-treated water, leak-tested, the carboy refilled with 9 liters RO water, then steam sterilized for 4 hours.

Bacteria

The facultative anaerobic bacterium, *Citrobacter freundii* a sulfate reducing bacterium, *Desulfovibrio gigas*, and an aerobic bacterium, *Pseudomonas aeruginosa* were used in the studies. *P. aeruginosa* (strain 8830), which was only used in the antibody studies, was obtained from Ananda Chakrabarty, University of Illinois at Chicago. Strain 8830 was selected due to its ability to produce alginate. The alginate matrix has been shown to impede oxygen transfer. *C. freundii* was obtained from M.W. Mittelman, University of Tennessee at Knoxville and was an environmental isolate from an active tubercle from a Tennessee Valley Authority pipeline that was identified by an Analytical Profile Index (API) 20E assay (Analytical Products Division, Sherwood Medical, Plainview, NY). *C. freundii* was a good candidate to study MIC on metal surfaces as it belongs to the Enterobacteriaceae family (Bergey's Manual of Systematic Bacteriology). Some members of this family have been implicated in MIC processes (25) and have been proposed to coexist with sulfate reducing bacteria (27,73,77). *D. gigas* (ATCC #19364) was obtained from the American Type Culture Collection (ATCC) Rockville, MD. It was used because of its unusually large size, and characteristic morphology allowing identification based on these features within the thin films. Sulfate reducing bacteria have been reported to cause or initiate MIC on stainless steel (42).

Suspended Cell Growth Studies

A growth curve was performed using *D. gigas* in the presence or absence of *C. freundii*. Replicate stopper-sealed flasks were filled with 10 strength EPRI medium as described below, amended with resazurin (0.1 mg/ml) and ascorbic acid (1 g/l) as a reducing agent, flushed with nitrogen, then autoclaved for 15 minutes. Modified Hutner salts and iron chloride were added after autoclaving. An inoculum of approximately 1×10^5 cells of *D. gigas*, verified via AODC, from a culture grown anaerobically for 14 days at 22°C was used. An inoculum of approximately 1×10^8 cells of *C. freundii* was used from a 24 hour batch culture of *C. freundii* grown in EPRI medium at 22°C. Samples of suspended cells were enumerated daily for 27 days via AODC on membrane filters as will be described. *C. freundii* was enumerated at 1000X magnification, while *D. gigas* was enumerated at 400X and differentiated from *C. freundii* on the basis of morphology (Figure 2).



Figure 2. Image showing *D. gigas* (large vibroid bacteria) and *C. freundii* (very small rods) stained with DAPI on the same membrane filter at 400X magnification.

Culture Medium

A culture medium (referred to as EPRI medium) containing approximately 45 ppm organic carbon, was used to encourage growth of bacteria on the coupon surface.

The medium contained the following components in 1 liter volume:

50 mg	sodium lactate
50 mg	sodium succinate
50 mg	ammonium nitrate
50 mg	sodium sulfate
50 mg	yeast extract
1.0 ml*	Modified Hutner salts (13, #2 in Appendix)
0.19 g	monobasic potassium phosphate
0.63 g	dibasic potassium phosphate
0.33 ml*	10mM ferric chloride

* Filter sterilized and added after autoclaving of other medium components

Enumeration of Suspended Bacteria via Membrane Filtration

Suspended bacteria were enumerated via membrane filtration. Membrane filtration was performed according to the method of Hobbie, *et al.*, (29). Black polycarbonate membrane filters with a diameter of 25 mm and a pore size of 0.22 μ m were used. Filters were mounted on Millipore filtration towers. No less than 2 ml of cell suspension was filtered. If the cell suspension to be filtered was less than 2 ml, filter-sterilized phosphate buffered water (0.19 g/l KH_2PO_4 , 0.63g/l K_2HPO_4) (PBW) was added to increase the volume to this minimum. Stains (acridine orange or DAPI) were added to the sample prior to filtration, allowed to react for 5 minutes, then filtered using 7 in. Hg vacuum supplied by a vacuum

pump. After staining, filters were removed and enumerated using an epifluorescent microscope equipped with an ocular counting grid. Calibrated areas for the grid at various magnifications are listed in Table 1. The filter area was 201.062 mm². At least 10 fields were counted per membrane. Average cell densities on the filter membrane were multiplied by the area counted and reported as cells per ml.

$$\frac{\text{Average cell density}}{\text{Grid}} \times \frac{\text{Filter area}}{\text{Grid area}} \div \text{volume filtered} = \text{cells/ml}$$

Table 1. Grid areas at various magnifications

Objective	Zoom	Scale in μm	Grid Area(mm ²)
20	1.0	215	.046200
40	1.0	110	.012100
100	1.0	44	.001936
100	1.25	35	.001225
100	1.5	29	.000841

Studies of Bacterial Colonization of Stainless Steel in the Aerobic Reactor

EPRI medium was prepared 10 times strength the aforementioned concentration in one liter of RO water, steam sterilized for 20 minutes, then incubated at 22°C for seven days. If no growth was visually evident after this time, the media was aseptically transferred by pumping through sterile silicone tubing into the 9 liters of sterile RO water (1:10 dilution) in the previously autoclaved reactor system. Modified Hutner salts and 10mM ferric chloride were filter

sterilized using a 0.2 μm pore size Acrodisc and transferred aseptically into the carboy. Upon filling the coupon chambers with EPRI medium and testing for contamination via AODC of the effluent, the system was inoculated as described below.

A one time inoculum of 1×10^7 cells of *P. aeruginosa*, verified by acridine orange direct count (AODC) was performed at the start of each experiment, as previously described. *P. aeruginosa* was used as an initial colonizing bacterium. A 24 hour batch culture of *P. aeruginosa* grown aerobically in EPRI medium at 22°C was used to inoculate the reactor. After 7 days, 1×10^5 cells of *D. gigas* were added to the reactor, verified via AODC. This inoculum was from a culture grown anaerobically for 14 days in 10 times normal-strength EPRI medium at 22°C. The experiment was continued aerobically for another 7 days, after which the reactor system was disassembled and the coupons removed and analyzed.

Table 2. Combinations of bacteria used for antibody studies.

Bacteria	Chamber 1	Chamber 2	Chamber 3	Chamber 4
<i>P. aeruginosa</i>	X	X		Sterile
<i>D. gigas</i>		X	X	Sterile

Upon termination of the experiment, each chamber was opened using a sterile razor blade, the culture media poured into a beaker, and the coupon removed with sterile forceps. The control coupon was always removed first, followed by the *D. gigas* only chamber. Three 50mm x 50 mm pieces from each coupon were cut for antibody staining. The remaining portion of the coupon was

Coupons were cut into four 5 cm long segments for various analyses. A segment was scraped to remove attached bacteria using a sterile cell scraper. This was done by wetting the scraper in a sterile dilution vial containing sterile PBW, then scraping one surface of the segment 20 times back into the same vial. Direct counts of the scrapings via AODC were performed along with viable plate counts on R₂A agar to enumerate *P. aeruginosa* and 3 tube most probable number (MPN) (3) in 10 strength anaerobic EPRI medium to enumerate sulfate reducing bacteria. MPN tubes were considered positive for SRB if they contained black iron sulfide precipitate. Another segment was stained directly for 5 minutes using a 1 µg/ml solution of DAPI to determine bacterial densities before scraping. At least 30 fields per segment were counted. Densities were recorded in cells/cm². Scraping efficiency was also determined by staining a segment after scraping with DAPI and enumerating as described above. The density of bacteria remaining on the coupon divided by the density of bacteria initially on the coupon multiplied by 100 then subtracted from 100 was the percent removed.

$$100 - \frac{\text{density of bacteria after scraping}}{\text{density of bacteria before scraping}} \times 100 = \% \text{ removed}$$

Evaluation of Fluorescent Antibodies Used to Detect SRB

A rabbit anti-SRB polyclonal antibody, supplied by Bioindustrial Technologies, Inc. (Grafton, NY), was used to detect SRB. Tetramethylrhodamine isothiocyanate (TRITC) labelled anti-rabbit goat IgG antibody (Sigma

Immunochemical, St. Louis, MO) was used as the fluorescent labelled conjugate. A 1:40 dilution for both the antibody and the conjugated antibody in filter-sterilized PBW was used (Dave Jewolski, Bioindustrial Technologies, personal communication). Fluorescence intensity was qualitatively recorded according to graded fluorescence ratings (64) using an immunofluorescent slide assay as described below.

Immunofluorescent slide assays (IFA) were done using multi-well cover slides with each well containing 20 μ l of suspended cells (62). The assay was modified as follows: bacteria were additionally stained with DAPI (1 μ g/ml) for 5 minutes, each incubation was done for 60 minutes, and conjugated antibody incubation was done in darkness. For each assay, a positive control was run with 2% formaldehyde-fixed SRB cell suspension obtained from Bioindustrial Technologies, Inc, Grafton, NY.

Antibody binding efficiency was performed qualitatively using IFA and quantitatively using the method of Hoff (30) on filter membranes as will be described. An epifluorescent microscope equipped with excitation at 320nm and 520nm, allowing the same field to be analyzed under two different fluorescent illuminations was used. Twenty fields were examined per sample. Fluorescing bacteria in each field were counted after excitation at the two wavelengths.

Fluorescent Antibody Staining to Determine Surface-Associated SRB

The three segments set aside for the fluorescent antibody staining were stained with 50 μ l of a 1:40 dilution of an anti-SRB rabbit polyclonal antibody for

1 hour in a humidified chamber at room temperature. After 1 hour had elapsed, the segments were rinsed two times with sterile PBW, then stained with 50 μ l of a 1:40 dilution of TRITC-labelled anti-rabbit goat IgG in humidified chambers at room temperature in darkness for 1 hour. After this time, segments were rinsed 2 times with PBW, then counterstained with 10 μ l of DAPI for 5 minutes. Segments were then rinsed and fluorescing bacteria in each field counted after excitation at the two wavelengths as previously described.

Studies of Bacterial Colonization of Stainless Steel in the Anaerobic Reactor

Culture Medium

The culture medium used for the bacterial colonization experiments was the same as the medium used for the aerobic studies except it was amended with 1.3 g/l sodium sulfite as a reducing agent (40) for anaerobic studies. EPRI medium was prepared 10 times the aforementioned concentration in one liter of RO water, steam sterilized for 20 minutes, then incubated at 22°C for seven days. If no growth was visually evident after this time, the media was aseptically transferred by pumping through sterile silicone tubing into the 9 liters of sterile RO water (1:10 dilution) in the previously autoclaved reactor system. Modified Hutner salts, 10mM ferric chloride, and sodium sulfite were filter sterilized using a 0.2 μ m pore size Acrodisc and transferred aseptically into the carboy. The reactor system was placed in an anaerobic chamber and flow initiated. Upon filling the coupon

chambers with EPRI medium and testing for contamination via AODC of the effluent, the system was inoculated as described below.

Inoculation of Reactor with Mono- and Mixed Populations

Reactors were inoculated with mono and mixed bacterial populations as follows: 1) One chamber with *D. gigas* 2) One chamber *C. freundii* 3) One chamber with *C. freundii* and *D. gigas* 4) One chamber maintained as a sterile control with the coupon exposed to sterile flowing culture media for the duration of the experiment (Table 3).

Table 3. Combinations of bacteria used for anaerobic experiments

Bacteria	Chamber 1	Chamber 2	Chamber 3	Chamber 4
<i>C. freundii</i>	X	X		Sterile
<i>D. gigas</i>		X	X	Sterile

A one time inoculum of 1×10^7 cells of *C. freundii*, verified by acridine orange direct count (AODC) was performed at the start of each experiment (described in membrane filtration section). Bacterial inoculation of chambers containing *C. freundii* was done with a 24 hour batch culture of *C. freundii* grown anaerobically in EPRI medium at 22°C. As *C. freundii* is a facultatively anaerobic bacterium, it was used in lieu of *P. aeruginosa* for the anaerobic experiments. After *C. freundii* chambers had reached steady state, determined by daily effluent samples enumerated via AODC and viable aerobic plate counts on MacConkey agar, a one time inoculum of 1×10^5 cells of *D. gigas*, verified via AODC, was carried out (No

statistical difference was noted [data not shown] when incubating *C. freundii* plated on MacConkey agar in aerobic versus anaerobic conditions). This inoculum was from a culture grown anaerobically for 14 days in 10 times normal-strength EPRI medium at 22°C. After this time, the bacterial densities in the effluent were monitored daily, as aforementioned, for another 9 days, after which the reactor system was disassembled and the coupons removed and analyzed.

A second set of experiments was performed whereby coupons were removed from the reactor and analyzed 16 days, 3 weeks, and 4 weeks after inoculation with bacteria as previously described. Three chambers contained *C. freundii* and *D. gigas*, while the fourth chamber was a sterile control. The control coupon was exposed to sterile culture media for the full length of the experiment (4 weeks).

Coupon Sampling

Sampling was done as previously described in the antibody studies except that the 50mm x 50 mm pieces from each coupon were cut under anaerobic conditions and placed immediately into liquid nitrogen for further analysis via scanning Auger electron spectroscopy. The remaining portion of the coupon was placed in a sterile petri dish for direct and viable microbial enumeration as described below.

Enumeration of Biofilm Bacterial Populations

Coupons were cut and enumerated as described in the fluorescent antibody studies except that viable aerobic plate counts of surface scrapings were done on MacConkey agar for facultative enumeration. One unscraped segment was also placed directly in liquid nitrogen for surface chemical analysis, as will be described. Another unscraped segment of the coupon was stained using a 1 $\mu\text{g/ml}$ solution of acridine orange for 5 minutes, then examined using a scanning confocal laser microscope to determine bacterial colonization patterns as described below.

Bacterial Colonization Pattern Analysis

Scanning Confocal Laser Microscopy

Scanning confocal laser micrographs were taken of coupons exposed to bacteria using a Bio-Rad[®] Model MRC-600 scanning confocal laser microscope (SCLM) equipped with a krypton/argon laser (Bio-Rad Microscience Division, Cambridge, MA). The SCLM was used in conjunction with an Olympus microscope (Model#BH2, Olympus Optical Co. Ltd., Japan), equipped with an Olympus D Plan Apo UV series 160/1.30 oil immersion objective. Ten randomly chosen fields were examined per coupon at 2000X. For each field, two images were saved as digitalized images (pic files) using the Bio-Rad[®] Comos software. One image was a reflected white light image of the stainless steel surface, showing grains and grain boundaries. The second image was an epifluorescent image of acridine orange-stained bacteria illuminated using 488 nm excitation, 505 nm

emission barrier filter.

Image Processing and Analysis

The images were processed using software developed by Gary Harkin and Paul Shope in the Department of Computer Science at Montana State University, Bozeman, MT. The images were processed independently in a series of steps. First, the x,y positions of bacteria for a particular field were determined by an automated estimation of each bacterial location in relation to the centroid of its perimeter. Each centroid was denoted by a red "X", with its x,y position saved in a data file. The bacterial image was cleared from the screen. Then, the reflected white light image of the same field was processed. The surface grains and grain boundaries of the stainless steel coupon were visually distinct when viewed microscopically using reflected white light. The two contrast with grains seen as white or lighter shades of grey while grain boundaries appear black or in darker shades of grey in each field of view. For each coupon examined, a digitized image of each field of view had been captured. A stored image consists of a set of ordered triples (X,Y,pixel shade=I). A histogram of the pixel shades is bimodal, so it is possible, via a shade cutpoint, to estimate the number of boundary pixels. The coupons were slightly curved due to the fact that they were physically cut using tin snips, so an area of interest was chosen by manually creating a polygon. This area of interest was bounded by a yellow line. The amount of black pixels corresponding to the area of grain boundaries was then calculated. The number generated was the percent of black pixels in the area of interest compared to the

total number of pixels within that same area. These numbers were then recorded as percent area of grain boundary (P_i). Once the area of interest had been selected, the matching file containing the x,y positions of each bacterial cell was overlaid on the surface image file. At this point, the X's lying outside the area of interest were deleted. Then, the color of the X's were manually changed, depending on the position of the X relative to a grain boundary. If an X was on or touching a grain boundary, it remained its original red color. If an X was on a grain, its color was changed to yellow. If an X was very near a grain boundary, but not on a boundary, it was changed to blue. Once all the X's on the area of interest were evaluated, the x,y position, corresponding pixel value, and numerical color number (red=0, yellow=1, blue=2) were saved on a separate file. From these data, the percent bacteria on grain boundaries (Q_i) compared to the percent area containing grain boundaries (P_i) could be estimated for each image (i). These data were then subjected to statistical analysis.

Hypotheses and hypothesis tests were carried out assuming that the deposition of bacteria on the grain boundaries within each field of view is governed by a Poisson process. If this is true then the following null hypothesis was proposed: H_0 = bacteria have no preference for attachment to grains over grain boundaries and therefore the probability of a bacterium attaching in a region (grain or grain boundary) is proportional to the area of that region. Chi-square values were calculated to test the hypothesis as described by the following equation:

$$\chi^2 = \sum_{i=1}^n \frac{(Q_i - P_i)^2}{\frac{P_i(1-P_i)}{Q_i/N_i}}$$

Q_i = percent bacteria within grain boundaries
 P_i = percent area containing grain boundaries
 N_i = total bacteria within area of interest
 i = image number

P-values were calculated to determine the probability of a $\chi^2_{(N)}$ (where N= number of images processed or degrees of freedom) value greater than the observed value $\chi^2_{(N)}$ given that the null hypothesis is true (the bacteria have no preference to attach to grain boundaries over grains). If the p-value was less than 0.05, then the null hypothesis was rejected and an alternative hypothesis (the bacteria have a preference for attaching to grain boundaries over grains) was accepted.

Energy Dispersive X-ray Spectroscopy (EDX)

Coupons recovered after termination of the first experiment and a coupon "as received" from the supplier were examined using a EDX/scanning electron microscope to determine if any changes in the composition of the stainless steel coupon had occurred as a result of bacterial colonization. Coupons were examined using a 15 kilovolt electron beam which penetrated approximately 0.2 μm into the stainless steel with a maximum penetration of 0.85 μm , based on a density of 8.03g/cm³ for 316L stainless steel (80). The elements oxygen, iron, nickel, chlorine, chromium, sodium, potassium, phosphorus were quantified from

each spectrum using standardless analysis routine on Norton software. Spectral data were gathered from grains and grain boundaries on each coupon. The beam was focussed on the feature of interest (grain or grain boundary) to allow data to be acquired from each feature independently. The magnifications required for these acquisitions were approximately 180,000X for grain boundaries, and 40,000X for grains. A bulk percentage for each respective element was determined by analyzing 3 grains and 3 grain boundaries using EDX on an as received passivated 316L stainless steel surface (Table 4) and coupons recovered at the end of the first colonization experiment.

Table 4. Average bulk elemental percentages of 316L SS as determined by EDX.

Element	Grain Bulk%	GrainB Bulk%	Avg Bulk %
Chromium	18.05±0.13	17.68±0.44	17.87
Iron	69.48±0.89	69.88±1.01	69.68
Nickel	10.09±0.66	9.76±1.03	9.93

Auger Electron Spectroscopy

An Auger electron spectrometer coupled to a computer interface was used to obtain surface chemical information on the coupons. Surfaces were sputtered using an ionized argon beam. Four sputtering pulses of 15 second intervals were carried out to determine changes in surface chemistry with depth, with a sputtering rate of 1 Å per second on pure silicon. Sputtering pulses were followed by Auger analysis at 2000X magnification of 3 fields, each covering an area of approximately 50 µm x 50 µm. Small spot Auger spectroscopy was implemented to gather

spectra from 0.79 μm^2 fields of 3 grains or 3 grain boundaries within the sputtered area. Raw elemental percentages were calculated for each spectrum by measuring the peak height (PH) of each respective element (e_i), dividing by a known sensitivity factor (SF) for that respective element, then normalizing to 100% (15).

$$e_i \text{ Raw elemental } \% = \frac{\frac{e_i PH}{e_i SF}}{\sum_{i=1}^n \frac{e_i PH}{e_i SF}} \times 100 \% \quad (3)$$

i = element 1, ..., element n

The elemental percentage from each of the following elements was calculated: silicon, phosphorus, sulfur, chlorine, potassium, carbon, nitrogen, oxygen, chromium, iron, nickel, and sodium. Auger electron kinetic energies (Ke) were used to determine peak locations on each spectrum for each element and the sensitivity factors (SF) were used to calculate elemental percentages as shown below in table 5.

Table 5. Kinetic energies and sensitivity factors used for elemental percentage calculations.

	Si	P	S	Cl	K	C	N	O	Cr	Fe	Ni	Na
Ke	82	130	153	180	235	252	380	505	525	605	750	980
SF	.35	.52	.80	1.0	.80	.20	.30	.50	.35	.20	.27	.22

Chromium to nickel and iron to nickel ratios were calculated for each spectrum collected after the fourth 15 second sputtering pulse (60 second total

spectrum collected after the fourth 15 second sputtering pulse (60 second total sputtering time).

Atomic Force Microscopy

A coupon exposed to bacteria was examined using an atomic force microscope (AFM) to determine depth and width of grain boundaries. This was done by Clint Callahan at Imaging Services, Santa Barbara, CA. The sample was imaged in air using a Nanoscope III multimode AFM (Digital Instruments, Santa Barbara, California) operating in contact mode. The AFM was equipped with a 450 μm silicon cantilever (Digital Instruments) with an imaging force of approximately 10^{-9} N. Three different fields were examined on the sample with three different AFM section analyses done for each field. An average vertical distance or depth was determined for the grain boundaries based on 24 vertical distance measurements from the top of a grain to the bottom of a grain boundary (see Figure 3). An average horizontal distance of width was determined based on 12 horizontal distance measurements from each side of a grain boundary.



Figure 3. Section analysis generated by atomic force microscopy of the 316L stainless steel surface. Arrow color in upper left depth profile corresponds to vertical distance data in lower right.

Statistical Analyses

The InStat computer program version 1.13 by GraphPAD software was used to statistically analyze the Auger raw elemental percentage, ratio, and adjusted percentage data. The statistical methods used were the two-tailed unpaired t test. Chi-square values were calculated for each data set from the bacterial colonization analysis data as previously described.

RESULTS

Fluorescent Antibodies

An anti-SRB polyclonal antibody was used as a means of determining where SRB were located on the stainless steel surface. The binding efficiency of the antibody was evaluated as previously described. The antibody was found to bind approximately 70% of the cells (± 1 SD) (See table 6), although the fluorescence was weaker than the positive control (data not shown). The antibody was also found to not cross-react with *C. freundii*.

Table 6. SRB antibody binding efficiency to *D. gigas*.

Fluorescent channel	Avg. cells/ 10 fields
DAPI	21.6 $\pm$ 5.8
TRITC	15.1 $\pm$ 2.6

Using the antibody SRB were found in aggregates in mixed biofilms grown aerobically in the reactor on a hand polished stainless steel surface (Figure 4).

Significance of Passivation

The passivation procedure previously described proved useful in removing autofluorescing material from the surface (Figure 5). Hand-polishing was originally done to eliminate this material from the surface. When the SRB antibody was introduced to the passivated surface, non-specific fluorescence

A



B

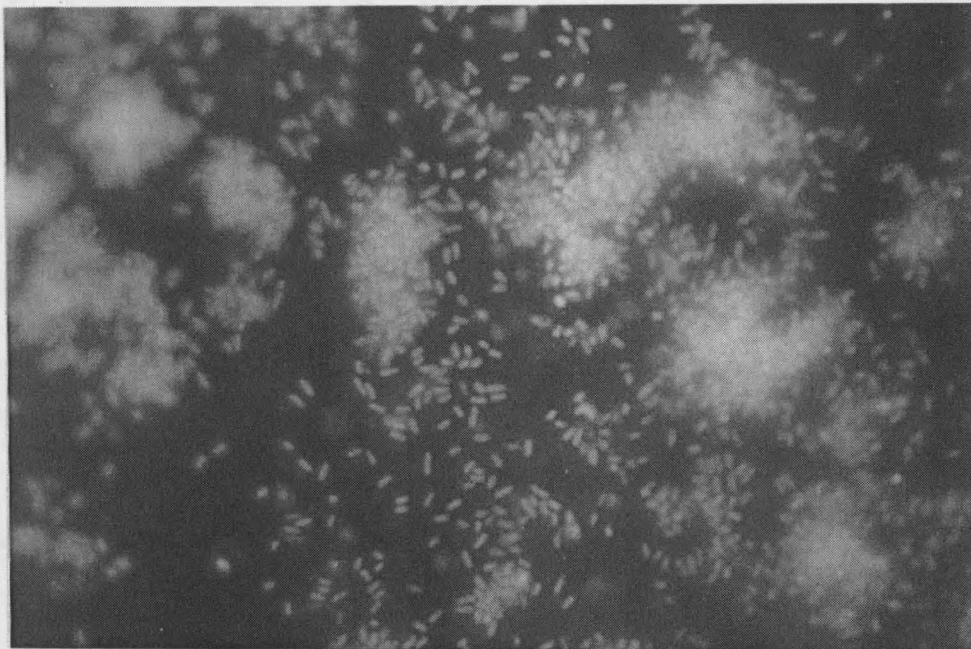
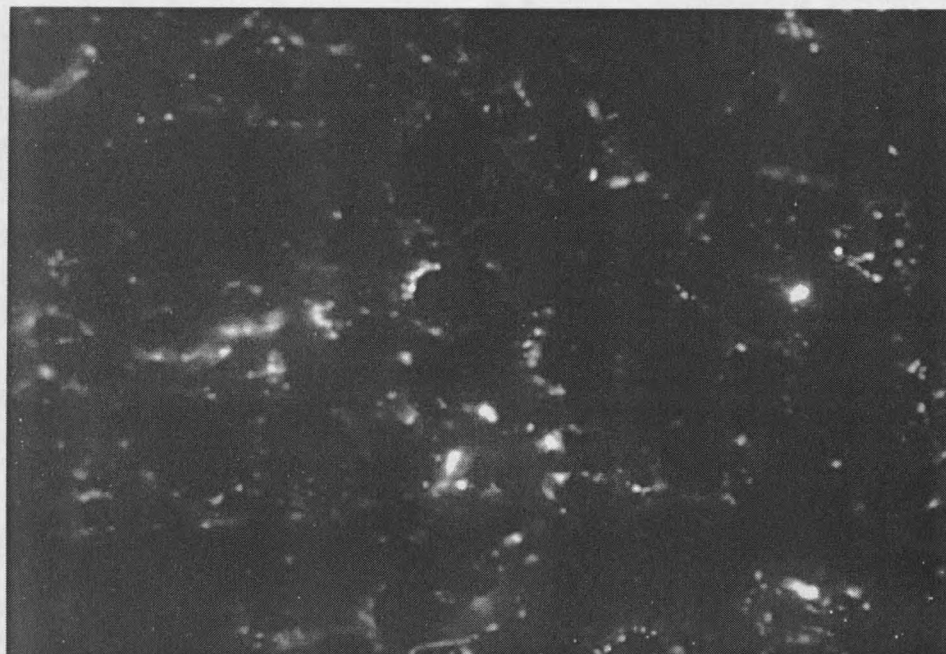


Figure 4. Biofilm containing *C. freundii* and *D. gigas* grown aerobically on hand polished 316L SS. Two illuminations of the same field (a) SRB stained with antibody (b) Total cells stained with DAPI. Magnification 1000X

A



B



Figure 5. Epifluorescent micrographs of unstained as-received 316L SS surface, comparing (a) non-passivated to (b) passivated. Autofluorescing material is very abundant in (a). Magnification 1000X.

resulted. The introduction of the antibody to the hand-polished surface did not have non-specific fluorescence. In order to more closely simulate industrial processes, for the purposes of this study, it was concluded that the SRB antibody could no longer be used. Therefore, in all further studies, SRB were identified by morphology rather than via fluorescent antibodies. *D. gigas* and *C. freundii* were shown to attach equally well and perhaps in slightly higher numbers (nearly one log) to the passivated stainless steel than to the hand-polished surface after 5 days in batch culture (Figure 6).

Growth of *D. gigas* in the Presence and Absence of *C. freundii*

Growth of *D. gigas* was measured in the presence and absence of *C. freundii* in anaerobic batch cultures. *D. gigas* (SRB) was shown to be increasing in number in the presence of *C. freundii*, whereas in the absence of *C. freundii*, there was no apparent increase in cell number after 27 days (Figure 7). Similar observations of the 2 species were recognized in the effluent monitored from the reactor (Figure 8). SRB were shown to increase in number on the coupon surface as well as the surfaces of the reactor system in the presence of *C. freundii* when compared to lines containing only SRB, however the increase was only approximately one log (Figure 9). *D. gigas* was also found within aggregates of *C. freundii* in biofilms grown in anaerobic batch culture on stainless steel coupons (Figure 10) however microcolonies of *D. gigas* were never observed in the anaerobic reactor experiments.

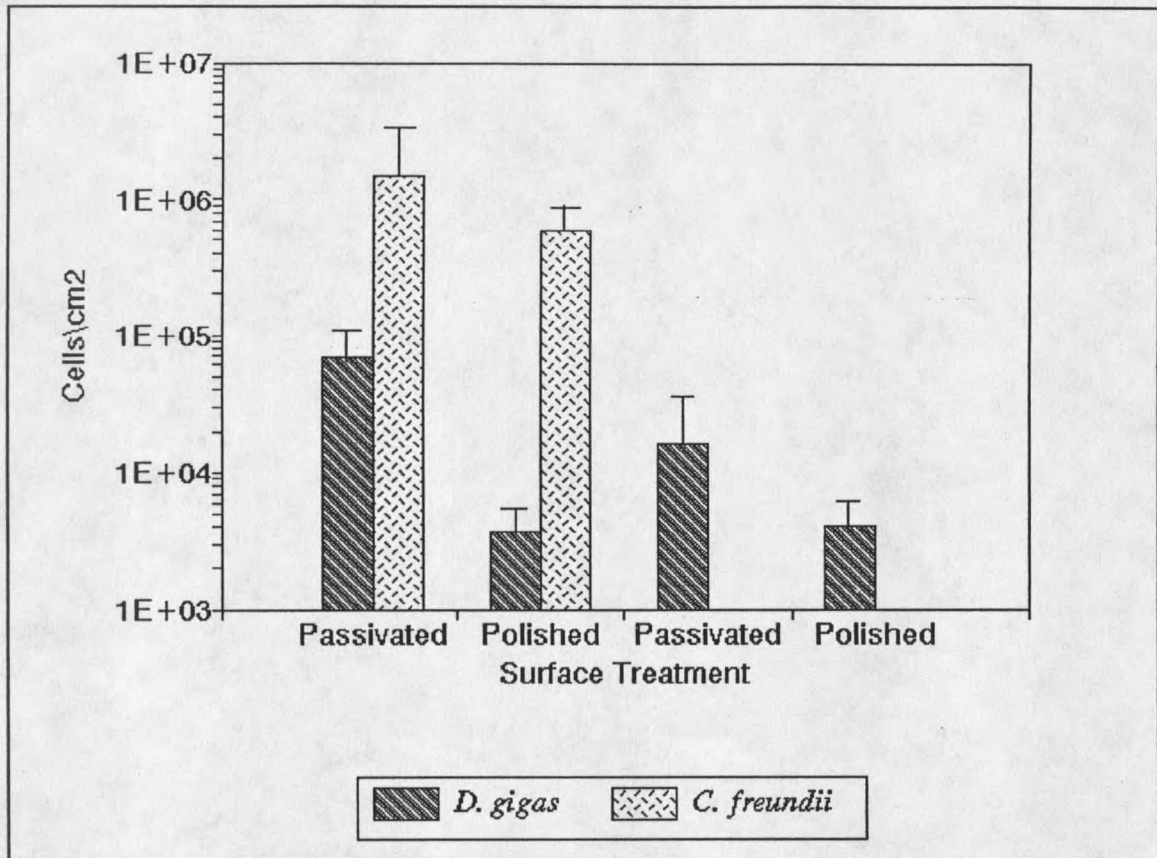


Figure 6. Bacteria attached to passivated and hand polished SS coupons suspended for 5 days in anaerobic batch cultures, 1 containing *D. gigas*, 1 containing *D. gigas*+*C. freundii* enumerated via DAPI DC.

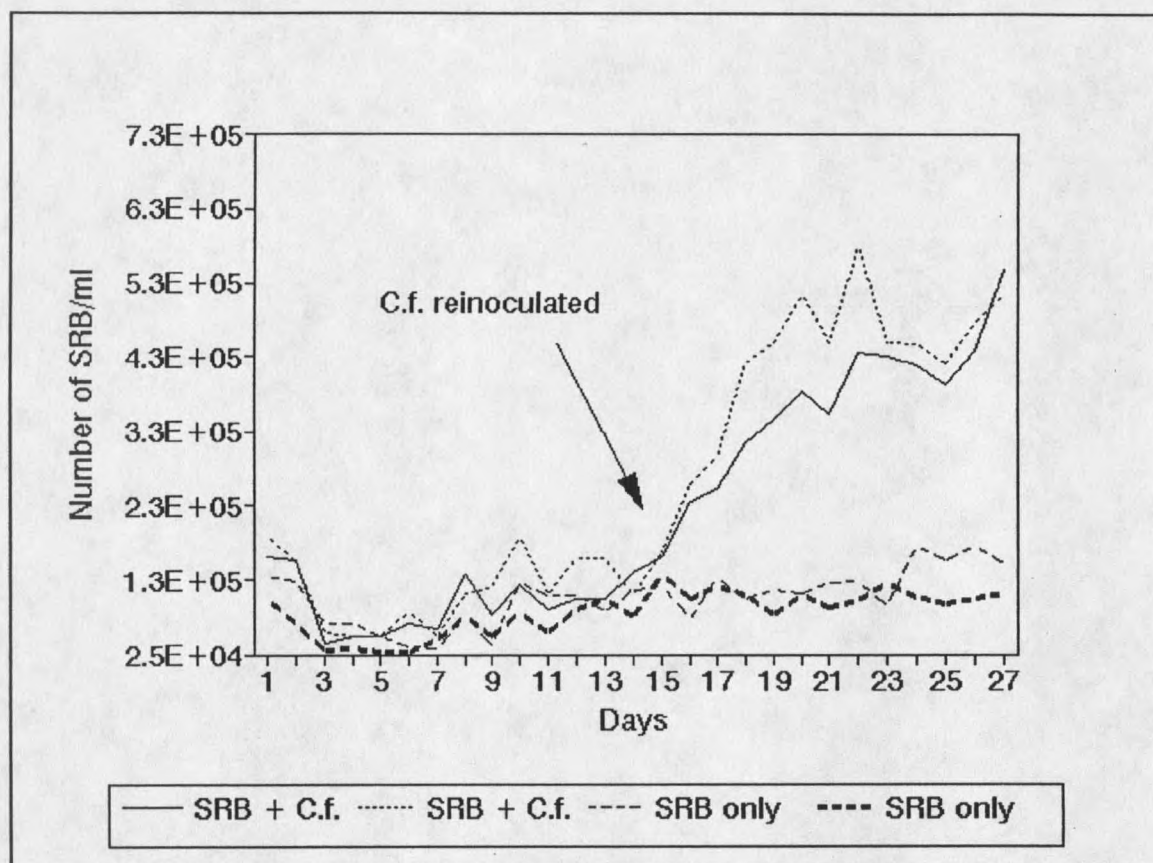


Figure 7. Anaerobic growth curve of *D. gigas* (SRB) in the presence and absence of *C. freundii* (C.f.) via DAPI direct count. Each line represents one batch culture flask at 22°C.

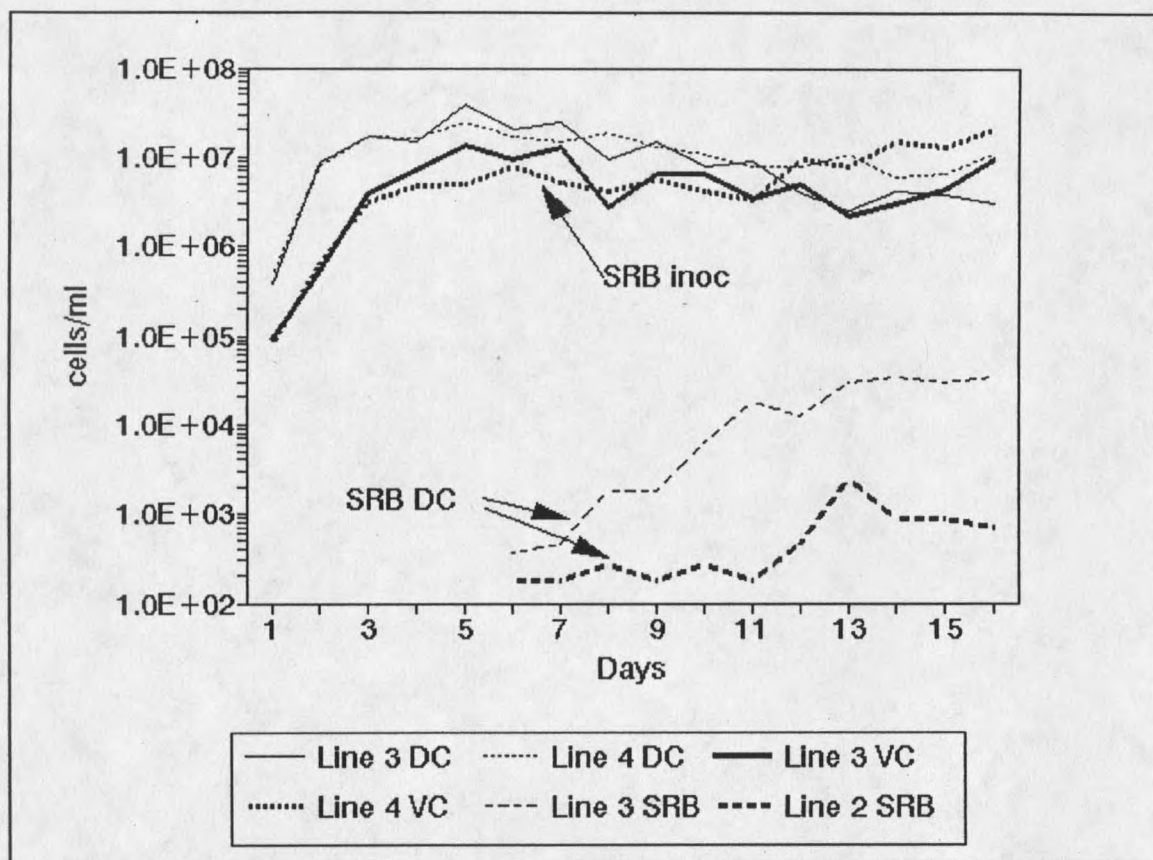


Figure 8. Viable (VC) and direct count (DC) of anaerobic reactor effluent. Line 2 contains only *D. gigas* (SRB), line 3 contains *D. gigas* (SRB) + *C. freundii*. (Cf), line 4 contains only *C.f.*

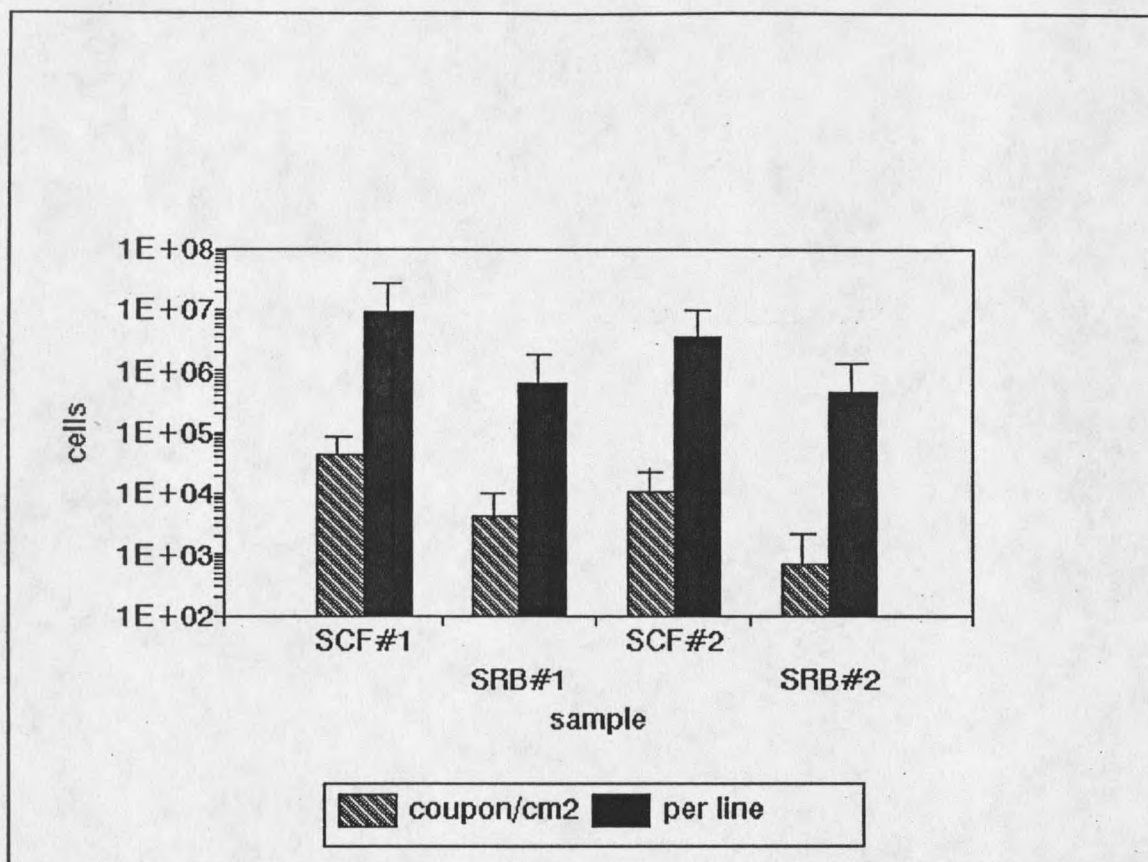


Figure 9. Direct counts of SRB on coupons and on total surface area of reactor. SCF (*C. freundii*+*D.gigas*), SRB (*D.gigas*). Results represent data from 2 experiments.



Figure 10. Epifluorescent micrograph of DAPI stained biofilm on 316L SS from batch culture containing *D. gigas* and *C. freundii*. Magnification 1000X.

Bacterial Colonization Patterns

Confocal laser microscopy of acridine orange stained bacteria that colonized the coupon surface and reflected white light images of the coupons from the reactor experiments were used to determine % area containing grain boundaries and % total bacteria within grain boundaries. If the bacteria are randomly distributed over the coupon surface then there is no surface influence on colonization. Thus, the percentage of total bacteria associated with the grain boundaries should be equal to the percent of the total area containing grain boundaries. The percent of total bacteria associated with the grain boundaries was significantly different ($p < 0.0001$) in the two sets of experiments presented in Figures 11, 12, 13, and 14. Of the 63 total images examined, the average percent area total containing grain boundary was 32.4, with a range of 11 to 47, while the average percent of total bacteria that were within the grain boundaries was 76.6, with a range of 61 to 90. The χ^2 test statistic was 11,626.71. When a p-value was calculated given the null hypothesis using this number with 63 degrees of freedom, p was equal to zero, within machine accuracy. Therefore, the null hypothesis that the bacteria have no preference of attaching to grain boundaries over grain was rejected and the alternative hypothesis that the bacteria have a preference for attaching to grain boundaries over grains was accepted. This indicates that there is very strong evidence that bacteria used in these experiments attach preferentially to grain boundaries.

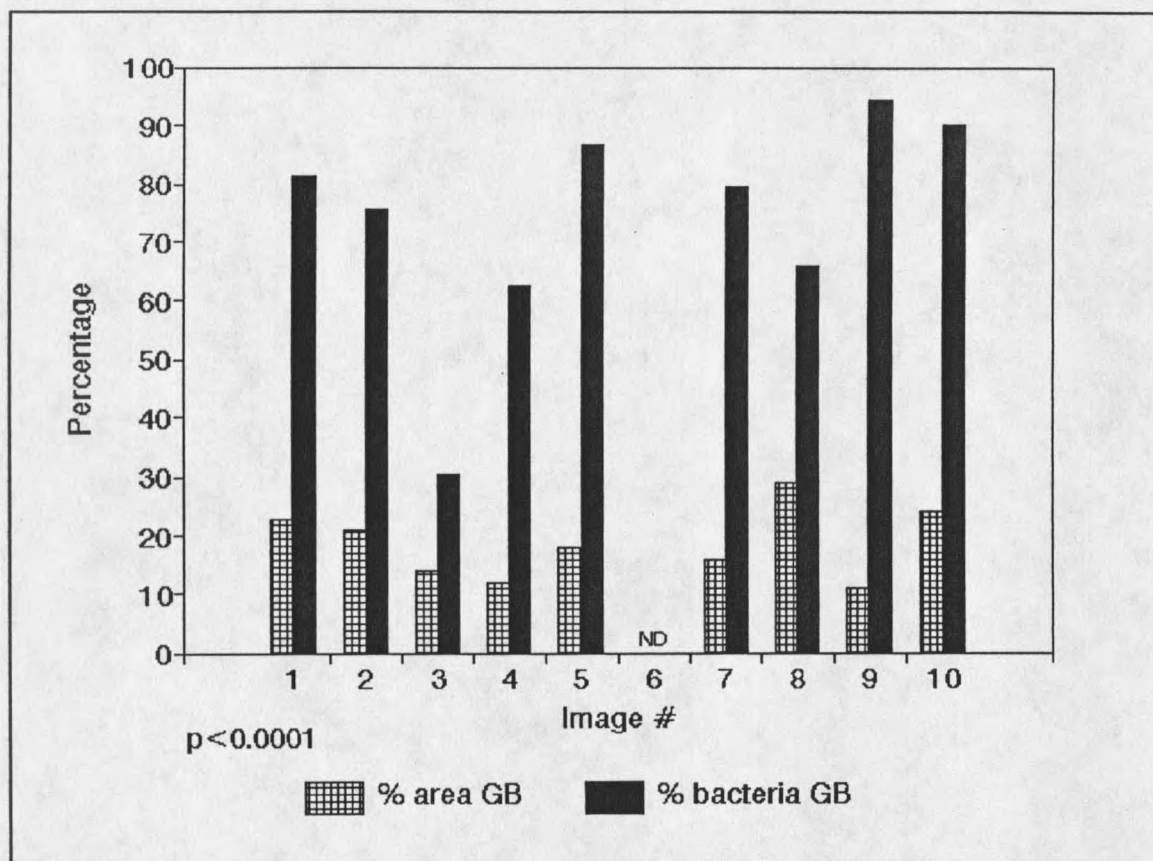


Figure 11. Total surface associated *C. freundii* located within grain boundaries (solid bars), compared to total area containing grain boundaries (hatched bars) after 16 days from experiment 1. ND, no data.

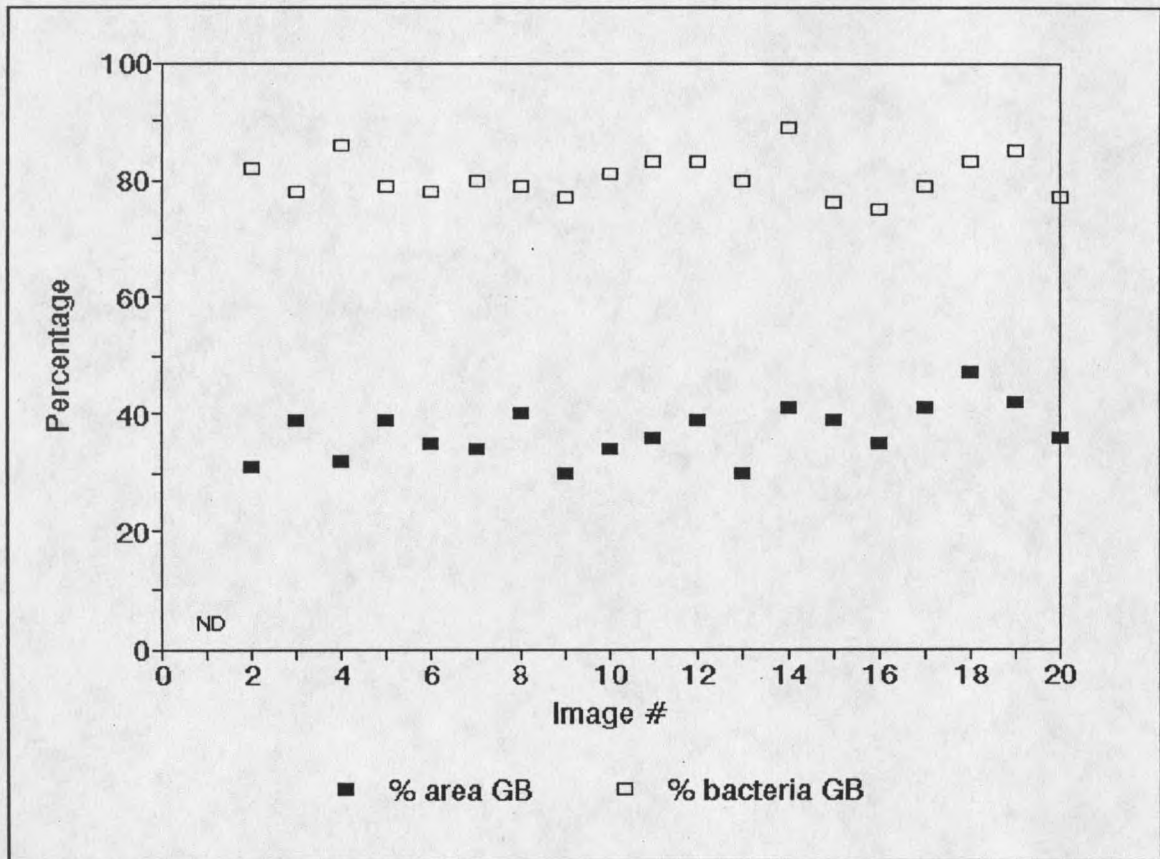


Figure 12. Percentages of total surface-associated *C. freundii* located at a grain boundary (GB) and percentage of total surface area (grains+GB) contributed by grain boundaries after 16 days in Exp. #2. ND, no data

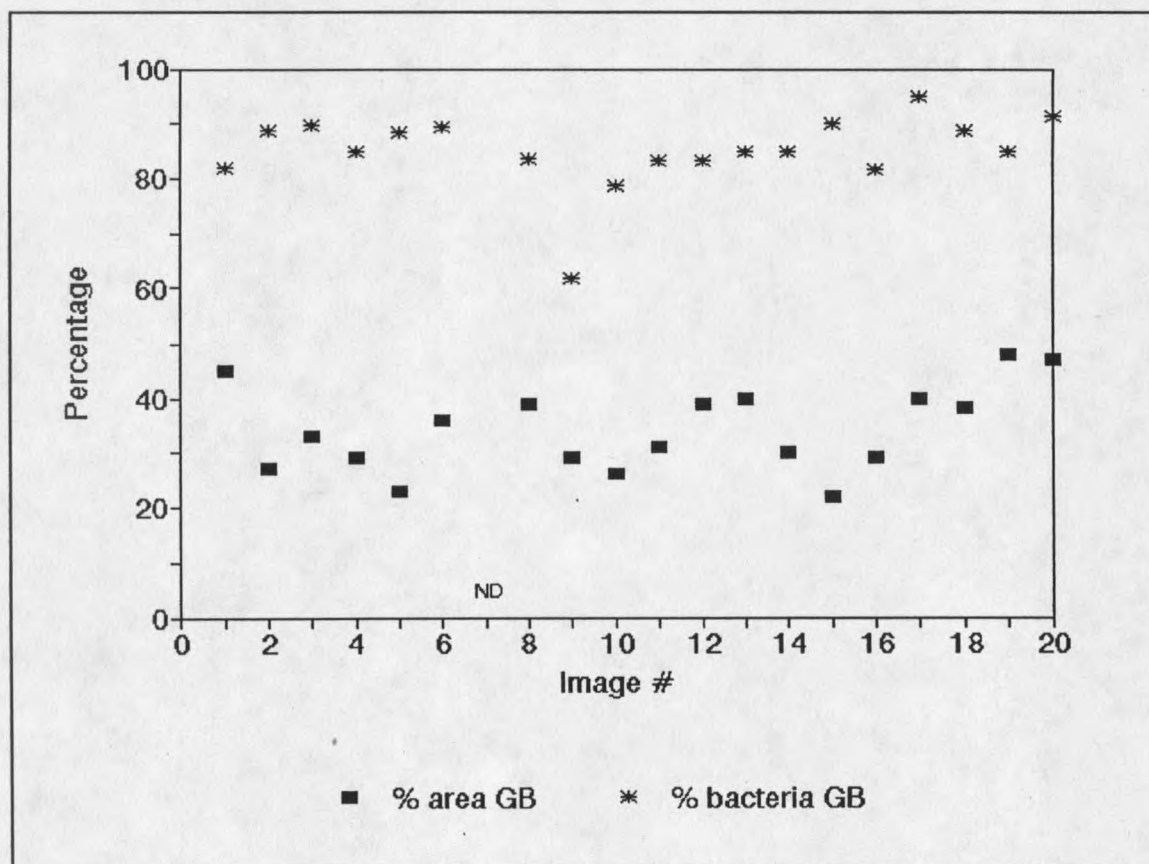


Figure 13. Exp #2. Percentage of total surface-associated *C. freundii* located at a grain boundary (GB) and percentage of total surface area (grains + grain boundaries) contributed by grain boundaries after 3 weeks. ND, no data

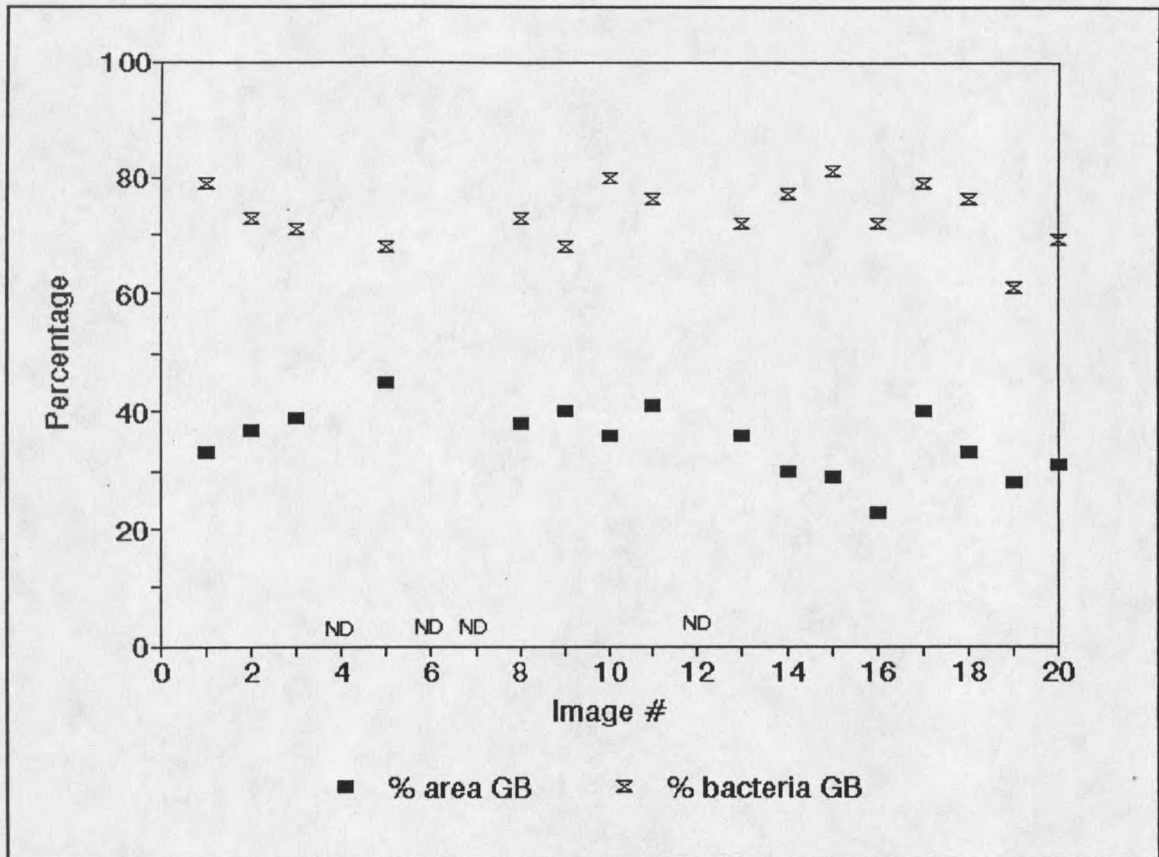


Figure 14. Exp #2. Percentage of total surface-associated *C. freundii* located at a grain boundary (GB) and percentage of total surface area (grains+GB) contributed by grain boundaries after 4 weeks. ND, no data

Surface Analysis

Energy Dispersive X-ray Spectroscopy (EDX)

EDX was employed to determine bulk stainless steel elemental percentages of chromium, iron, and nickel after passivation prior to bacterial exposure. As penetration depth via EDX was determined to be approximately 0.8 μm into the stainless steel, the elemental percentages recorded by EDX were compared to values recorded via Auger electron spectroscopy (AES). AES values were reported after sputtering for 60 seconds (sputtering rate of approximately 1 $\text{\AA}$ per second in pure silicon) and hereafter referred to as surface. Literature values of bulk percentages (80) were also compared in 316L stainless steel to determine if surface percentages were different from bulk values. As shown in Table 7, surface percentages of chromium are approximately 55% bulk values in both grains and grain boundaries.

Table 7. Elemental percentages of chromium, nickel, and iron in 316L stainless steel. Bulk grain (BG) and bulk grain boundary (BGB) measured by EDX, surface grain (SG) and surface grain boundary (SGB) measured by AES, literature values reported as nominal compositions in percentages (80).

Element	B G	B GB	S G	S GB	Literature Val.
Cr	18.1	17.7	10.7	10.4	16-18
Ni	10.1	9.8	10.2	11.2	10-14
Fe	69.5	69.9	63.9	53.9	68-74

Figure 15 depicts a conceptual model of the stainless steel prior to bacterial colonization and attack of the surface. The chromium-depleted zone is located

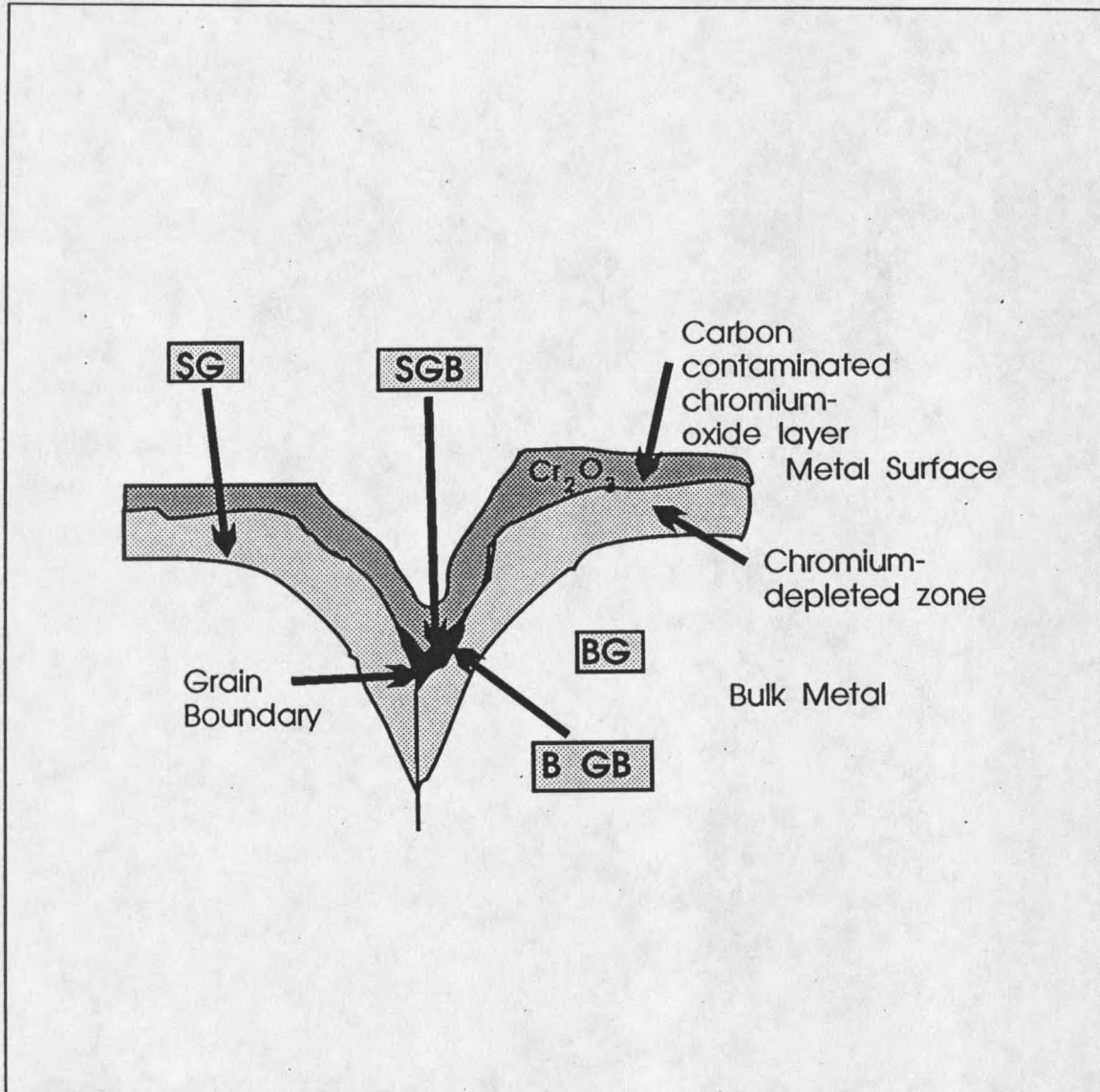


Figure 15. Conceptual model of stainless steel prior to bacterial colonization showing EDX and AES sampling regions. SG-Surface grain, SGB-surface grain boundary, BG-bulk grain, BGB-bulk grain boundary.

beneath the carbon-contaminated chromium-oxide layer. This zone depleted in chromium exists due to the formation of the chromium-oxide layer above. The chromium-depleted zone was the area measured by AES after removal of the chromium oxide layer by sputtering. The bulk metal was measured by EDX.

Scanning Auger Electron Spectroscopy (AES)

Small spot AES was used to analyze the surfaces of passivated stainless steel coupons exposed and unexposed to bacteria to determine if the bacteria had changed the surface elemental chemistry. A scanning electron micrograph of each field was generated, which was used to center the 2500 Å diameter beam on either a grain or a grain boundary (Figure 16).

Preliminary Auger spectral data indicated that the presence of the biofilms on the stainless steel may have caused some elemental changes to occur within the grain boundaries when compared to the grains. Both chromium and iron appeared to have been depleted within the grain boundaries when compared to the grains on the coupon surfaces exposed to bacteria (Figures 17, 18, 20, and 21). On the control stainless steel surface, such differences were not evident. On the other hand, nickel appeared to be unaffected by bacterial activities as there was no apparent difference in its percentage when comparing the grain and grain boundary information for all coupons for each of the two experiments (Figures 19 and 22).

In Figures 23 and 24, the chromium to nickel ratio was calculated for each coupon after 60 total seconds of sputtering. From the data in Table 7,

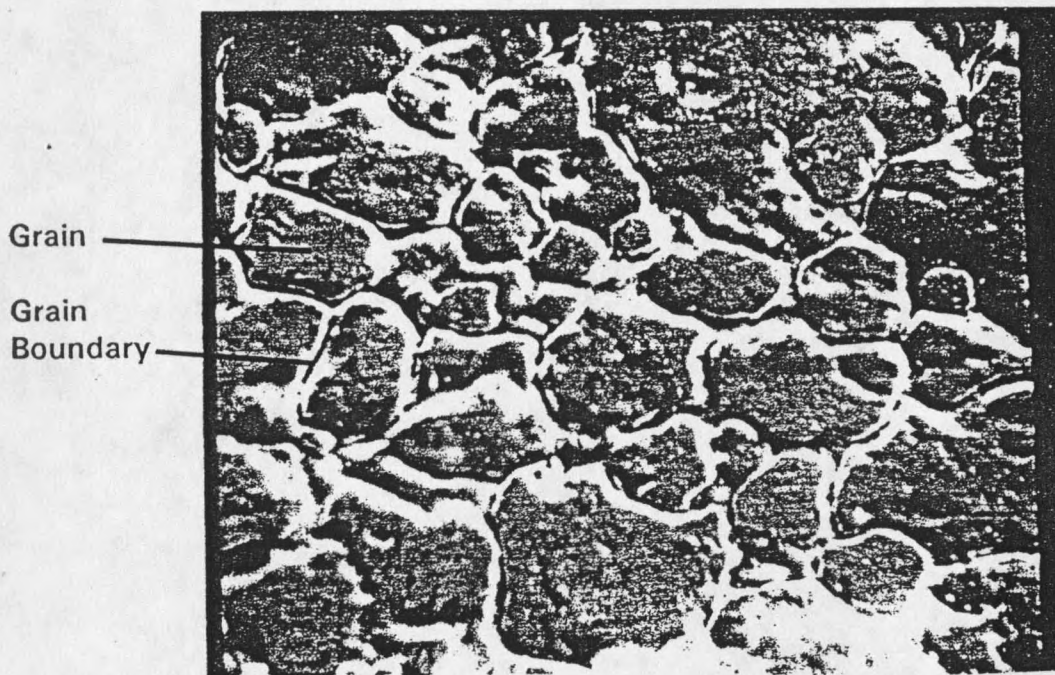


Figure 16. Scanning electron micrograph showing grains and grain boundaries on stainless steel surface. Magnification 2000X.

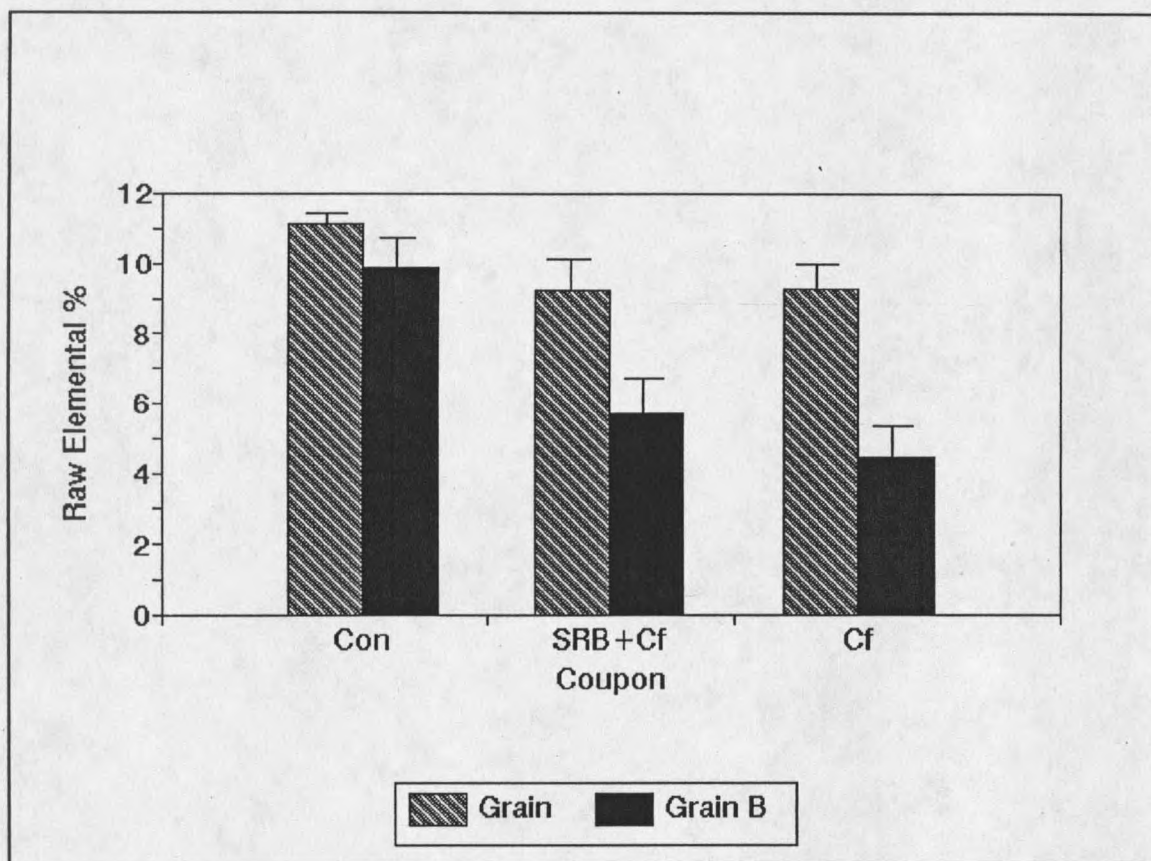


Figure 17. Abundance of chromium relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to *C. freundii* (Cf) with or without *D. gigas* (SRB) based on small-spot AES after 60 sec sputter time.

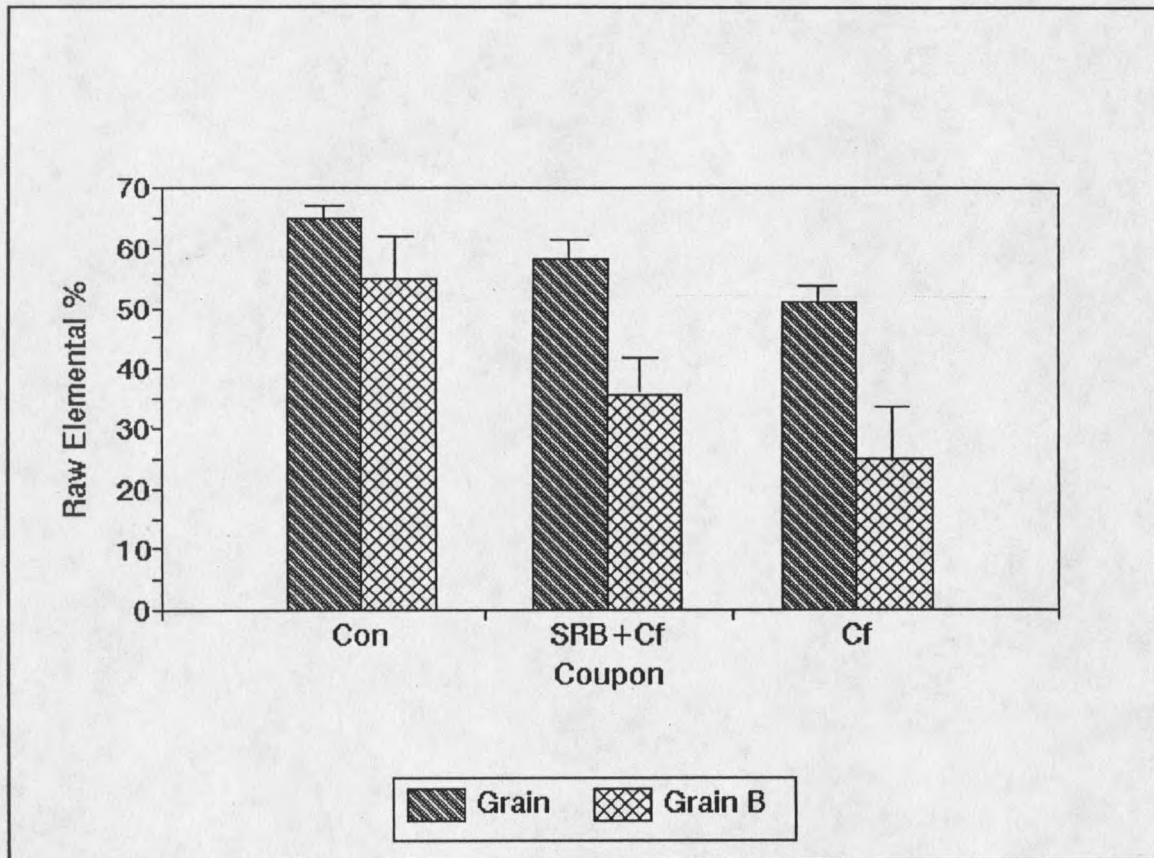


Figure 18. Abundance of iron relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to *C. freundii* (Cf) with or without *D. gigas* (SRB) based on small-spot AES after 60 sec sputter time.

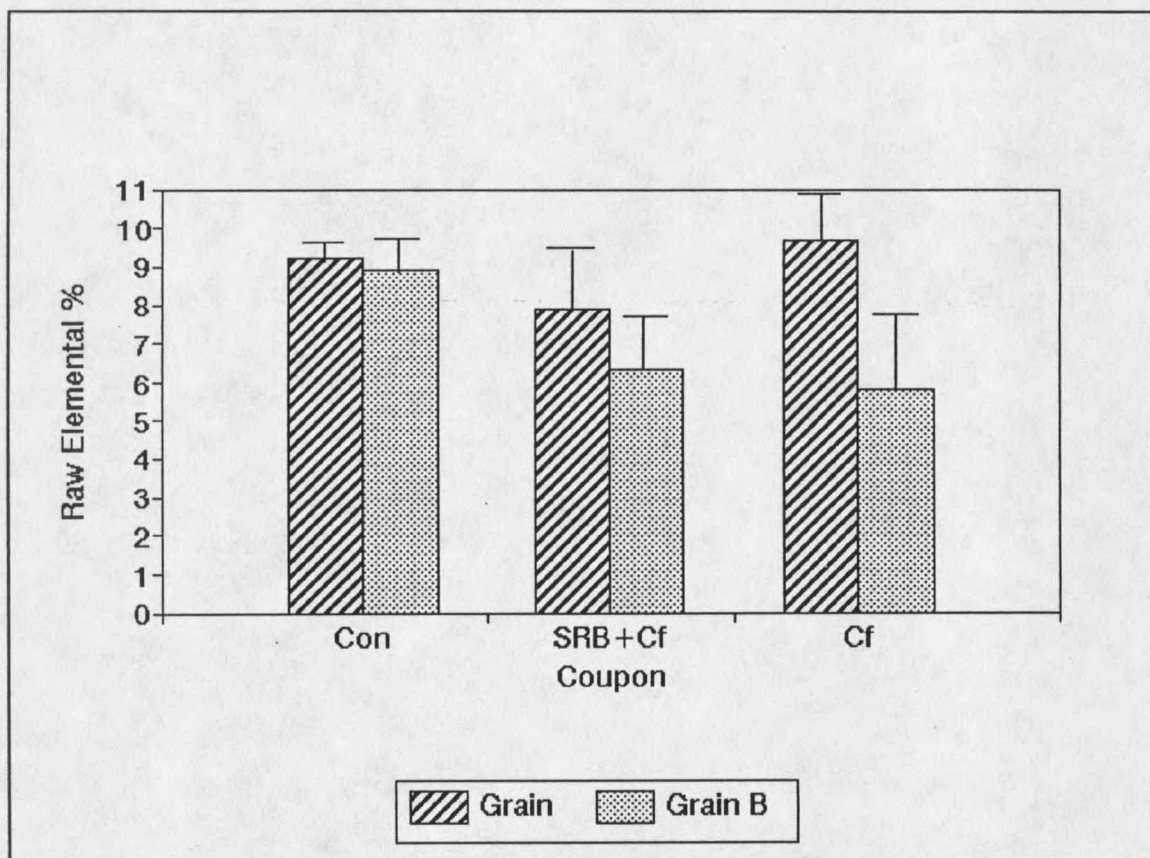


Figure 19. Abundance of nickel relative to all other elements in grains and grain boundaries on SS coupons exposed to sterile medium (Con) or *C. freundii* (Cf) with or without *D. gigas* (SRB) as determined by small-spot AES after 60 s sputtering.

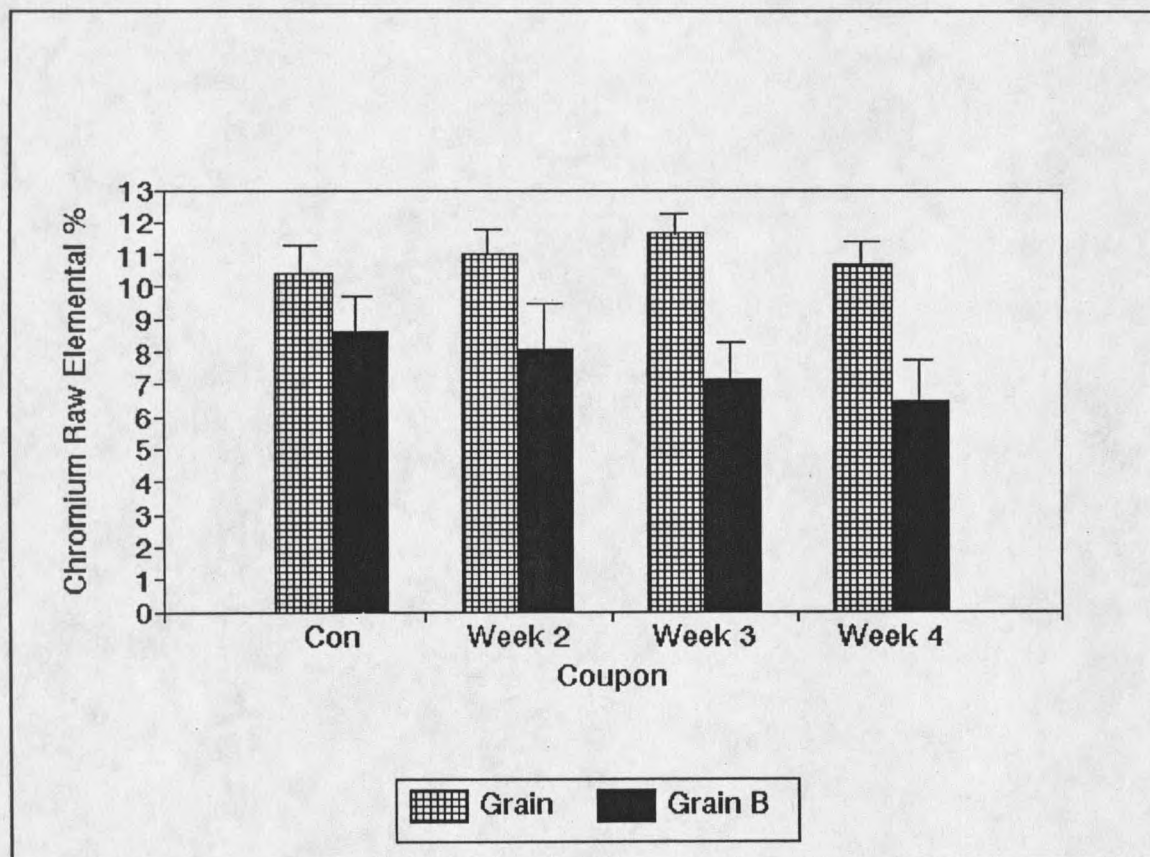


Figure 20. Abundance of chromium relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium(Con) or to *C. freundii*+*D. gigas* after 2, 3, or 4 weeks based on small spot AES after 60 s sputtering time.

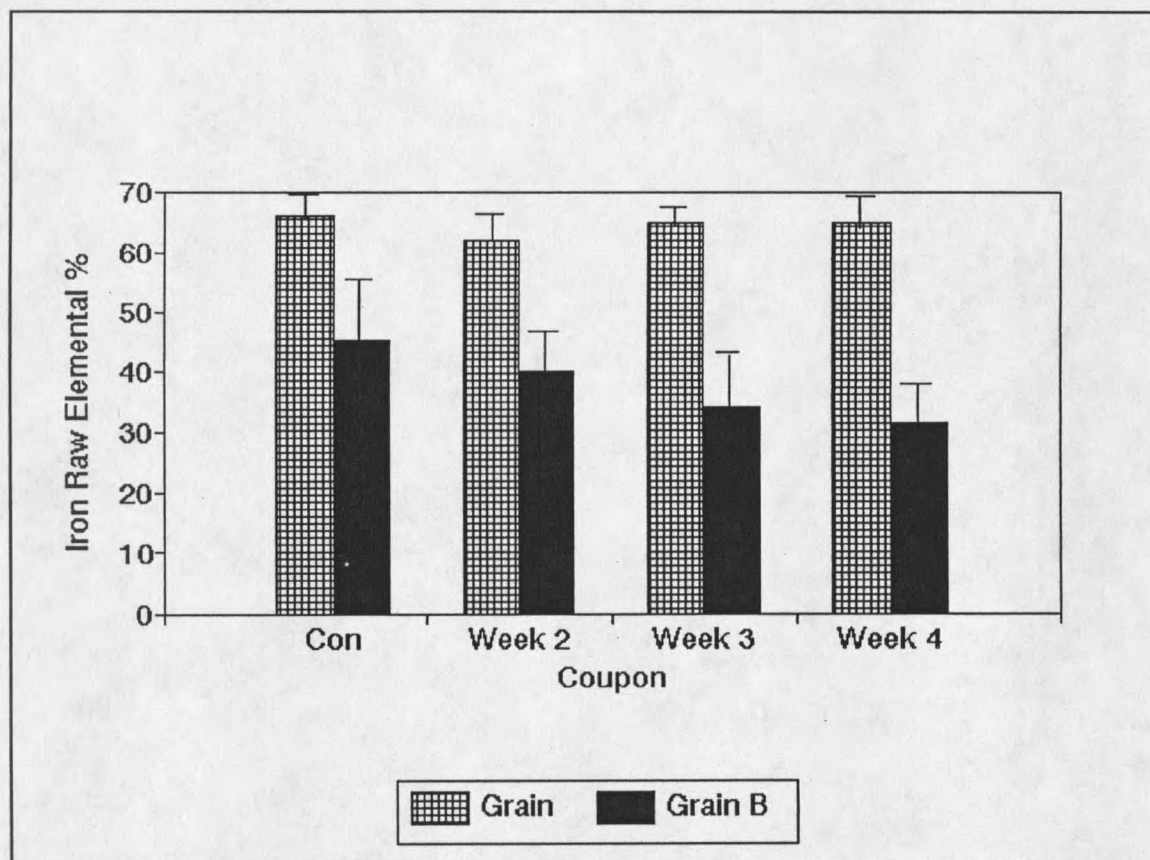


Figure 21. Abundance of iron relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium(Con) or to *C. freundii*+*D. gigas* for 2, 3, or 4 weeks based on small-spot AES after 60 sec sputter time.

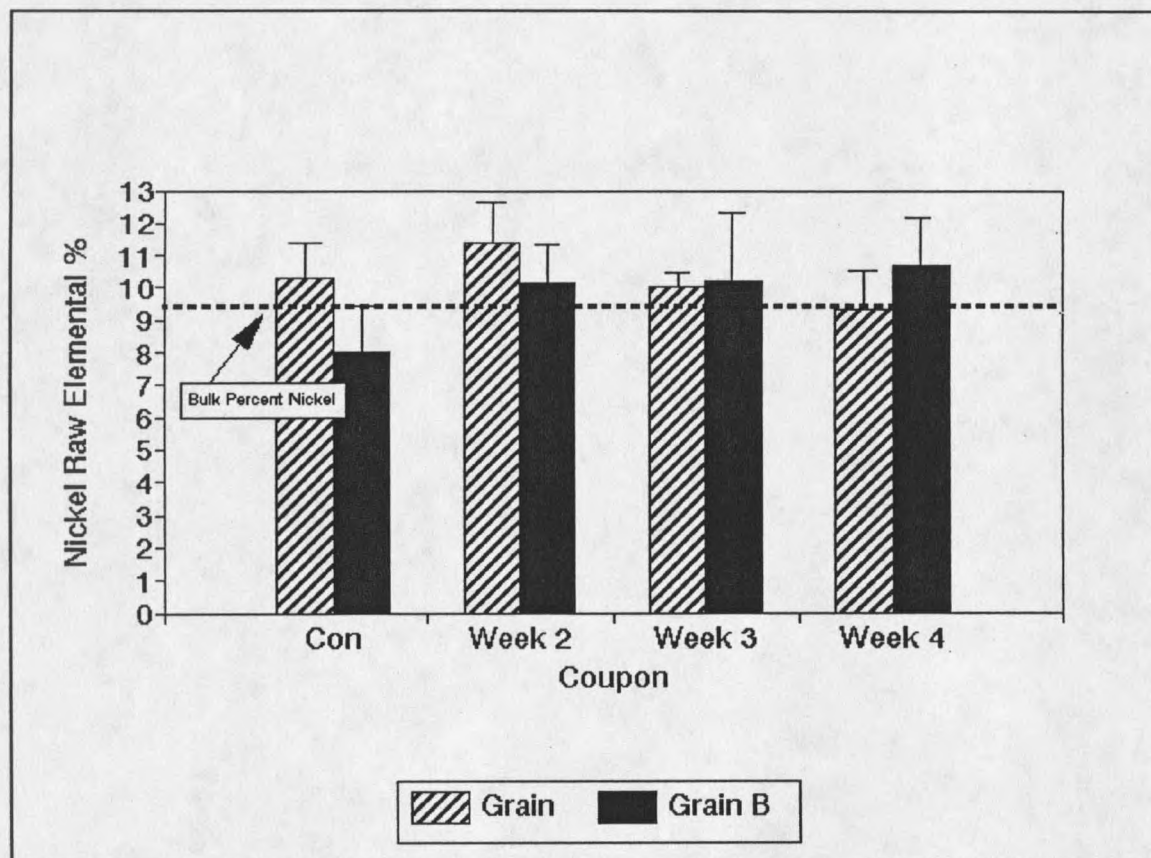


Figure 22. Abundance of nickel relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium(Con) or to *C. freundii*+*D. gigas* after 2, 3, or 4 weeks based on small-spot AES after 60 sec sputter time.

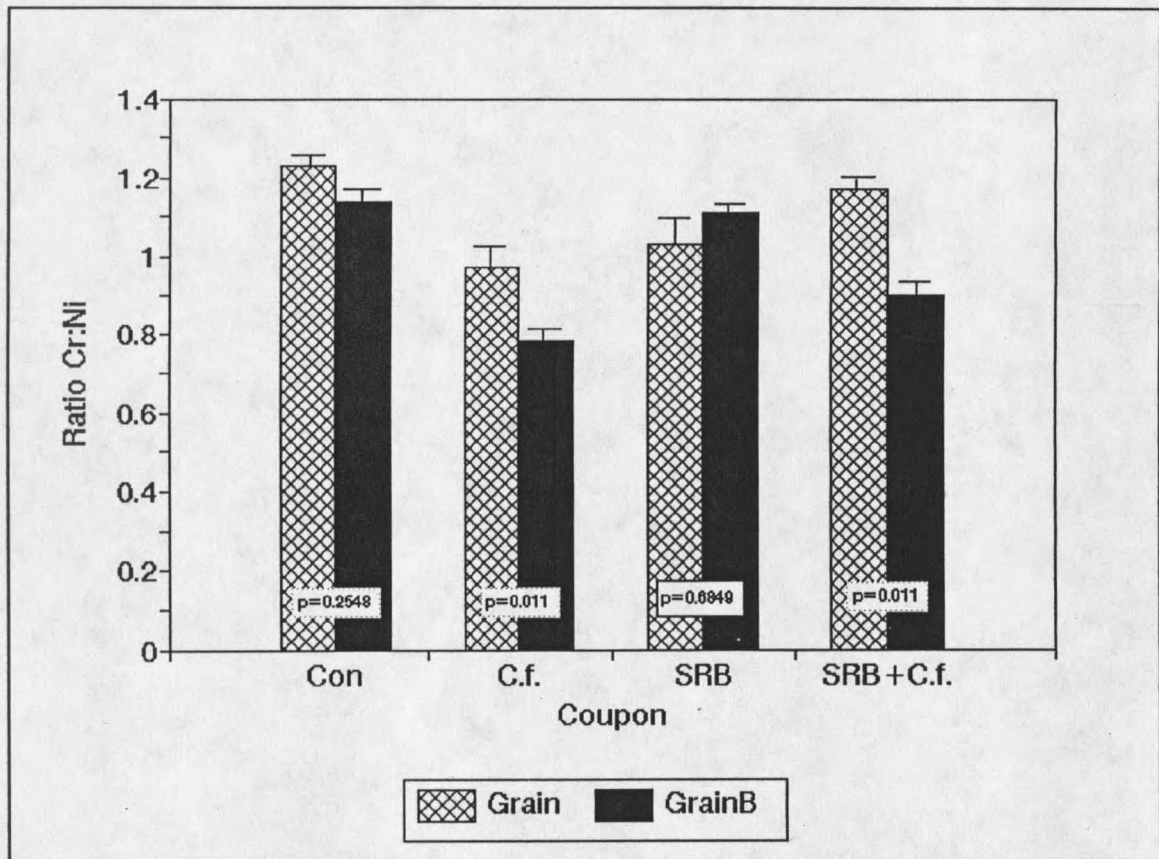


Figure 23. Ratios of chromium to nickel in grains and grain b. of SS coupons exposed to sterile medium (Con) or *C. freundii* (Cf) with or without *D. gigas* (SRB) as determined by small-spot AES after 60 s sputtering time.

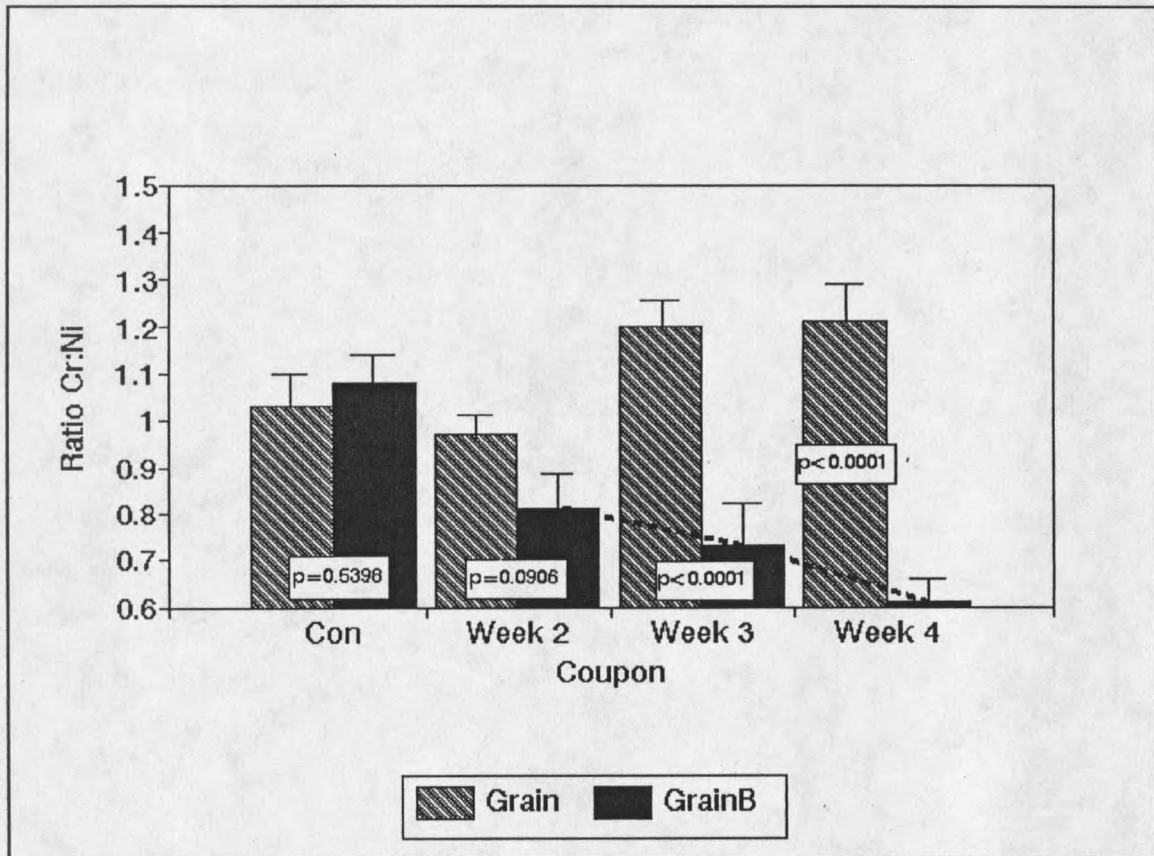


Figure 24. Ratios of chromium to nickel in grains and grain b. of SS coupons exposed to sterile medium (Con) or *C. freundii*+*D.gigas* for 2, 3, or 4 weeks as determined by small spot AES after 60 s sputter.

it was determined that 60 second sputter time is necessary to study the interface of the chromium oxide passive film and the bulk matrix and that a chromium depleted zone does occur after the passive layer (Cr_2O_3) is removed by sputtering (Figure 15). The passive layer was removed for this surface analysis to remove carbon contamination deposited during sample manipulation. Based on a 95% confidence interval, with a p value < 0.05 , the means of the ratios for coupons exposed to *C. freundii* were significantly lower in the grain boundaries than in the grains. On the control coupon and the coupon exposed to *D. gigas* only, however, there was no significant difference between the chromium to nickel ratios for grains and grain boundaries. The chromium to nickel ratio was not significantly different in grains and grain boundaries after 4 weeks exposure to sterile culture media. This indicates that the presence of *C. freundii* rather than *D. gigas*, led to depletion of the chromium relative to nickel in the grain boundaries.

In Figure 25, the iron to nickel ratio was calculated for each coupon after 60 total seconds of sputtering. The only coupon that had a significant decrease in the iron:nickel ratio was the coupon that had been exposed to both *C. freundii* and *D. gigas*. This seems to indicate that the presence of both *D. gigas* and *C. freundii* was necessary to cause a depletion of iron relative to nickel in the grain boundaries. This observation was further supported in Figure 26, where a trend in the depletion of iron relative to nickel was observed over time on the surface of the stainless steel coupons exposed to *D. gigas* and *C.*

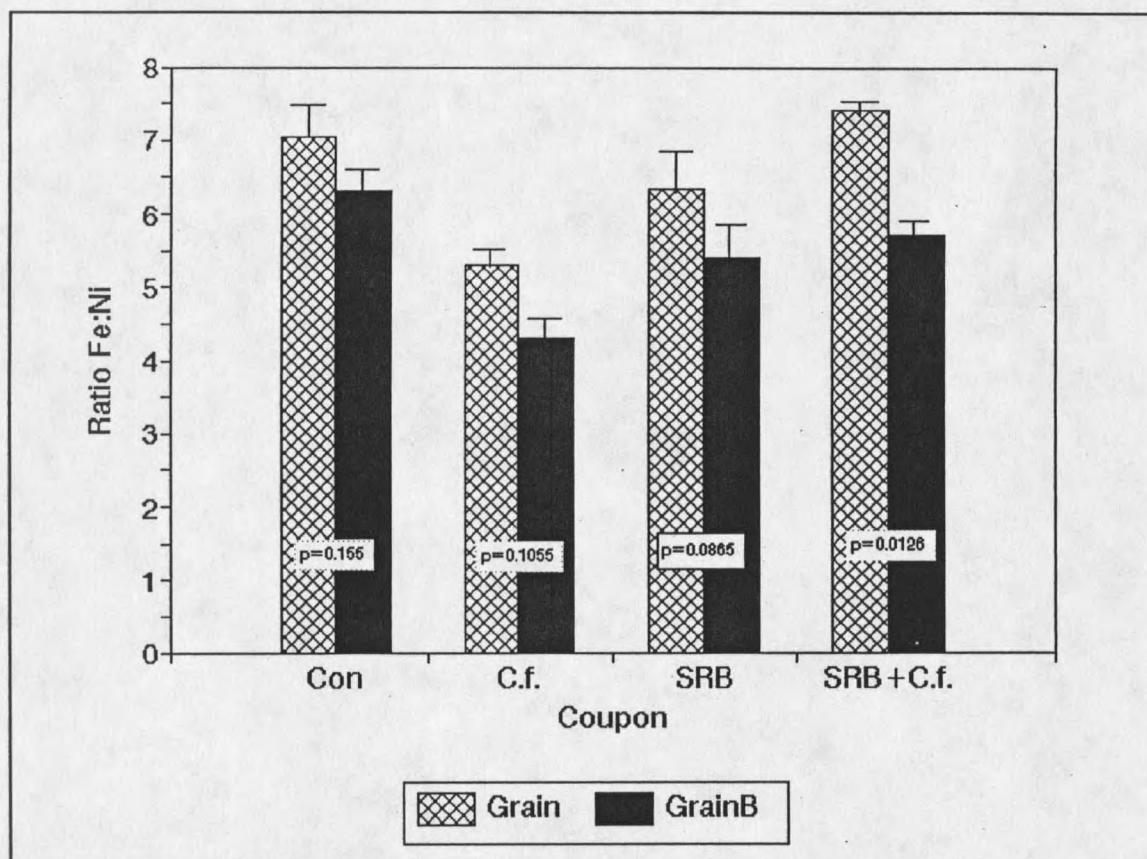


Figure 25. Ratios of iron to nickel in grains and grain b. of SS coupons exposed to sterile medium (Con) or *C. freundii* (Cf) with or without *D. gigas* (SRB) as determined by small spot AES after 60 s sputter time.

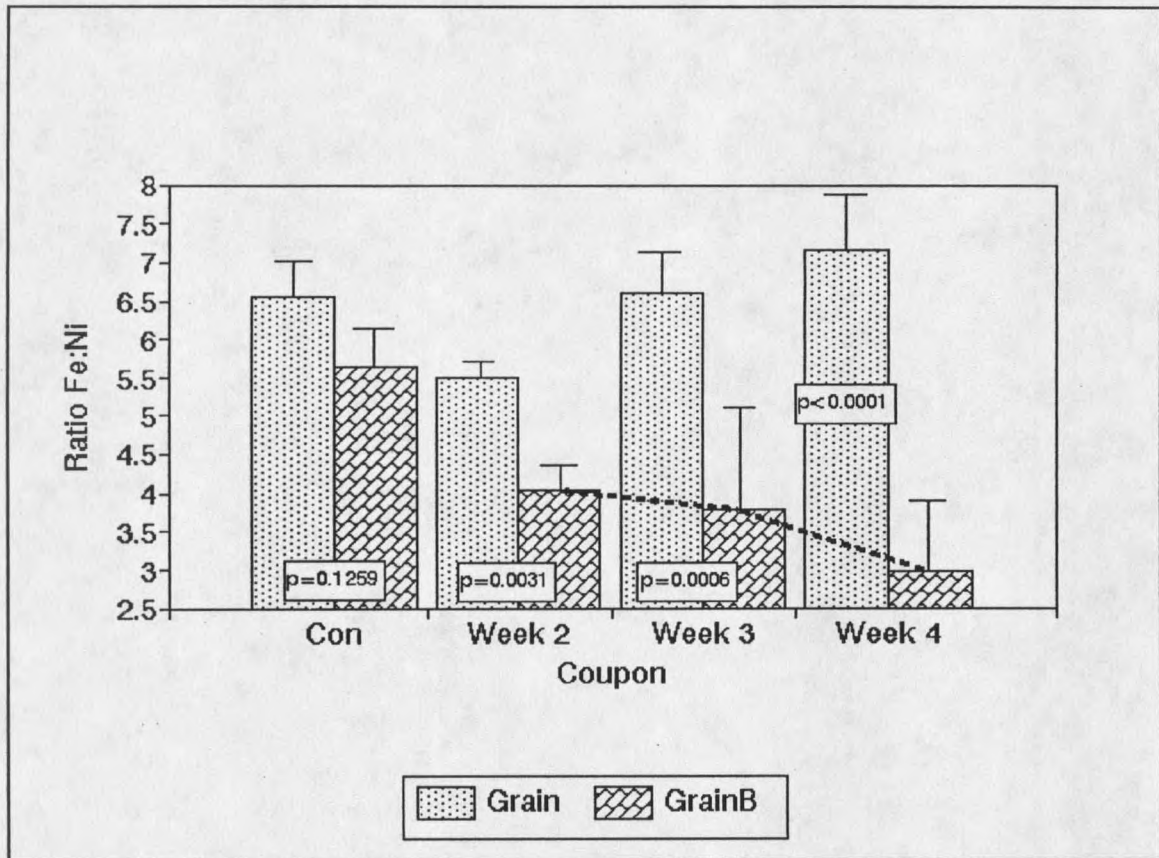


Figure 26. Ratios of iron to nickel from grains and grain b. of SS coupons exposed to sterile medium (Con) or to *C. freundii*+*D. gigas* for 2, 3, or 4 weeks as determined from small spot AES after 60 s sputter.

freundii. Furthermore, the control showed no significant change in the ratio when comparing grains and grain boundaries. This seemed to indicate that the presence of the bacteria may have caused the depletion of chromium and iron preferentially over nickel in the grain boundaries and that the longer the stainless steel was exposed to the bacteria, the greater the depletion.

Analysis of Non-alloy Elements

Non-alloy elements were defined as those elements which could not have originated from the stainless steel, but were still detected by AES. More than likely, most of these elements originated from the culture media or the biofilms, or both. In an effort to determine why the bacteria may be preferentially attaching to the grain boundaries, data was tabulated from the spectra for these elements. The first non-alloy element to be analyzed was nitrogen. Nitrogen data (Figure 27), indicated that both coupons that were exposed to bacteria showed significantly higher amounts of nitrogen in the grain boundaries than in the grains, whereas on the control, there was no significant difference. This indicates that the elevated levels of nitrogen in the grain boundaries of the coupons exposed to the bacteria may be the result of bacterial presence within the grain boundaries rather than abiotic deposition of nitrogen from the medium. The next non-alloy element to be analyzed was sulfur. After 60 total seconds of sputtering to remove carbon contamination deposited during sample manipulation, the concentration of sulfur in the grain boundaries was significantly higher in all coupons when compared to the grains (Figure 28). This is of particular significance in the sterile control

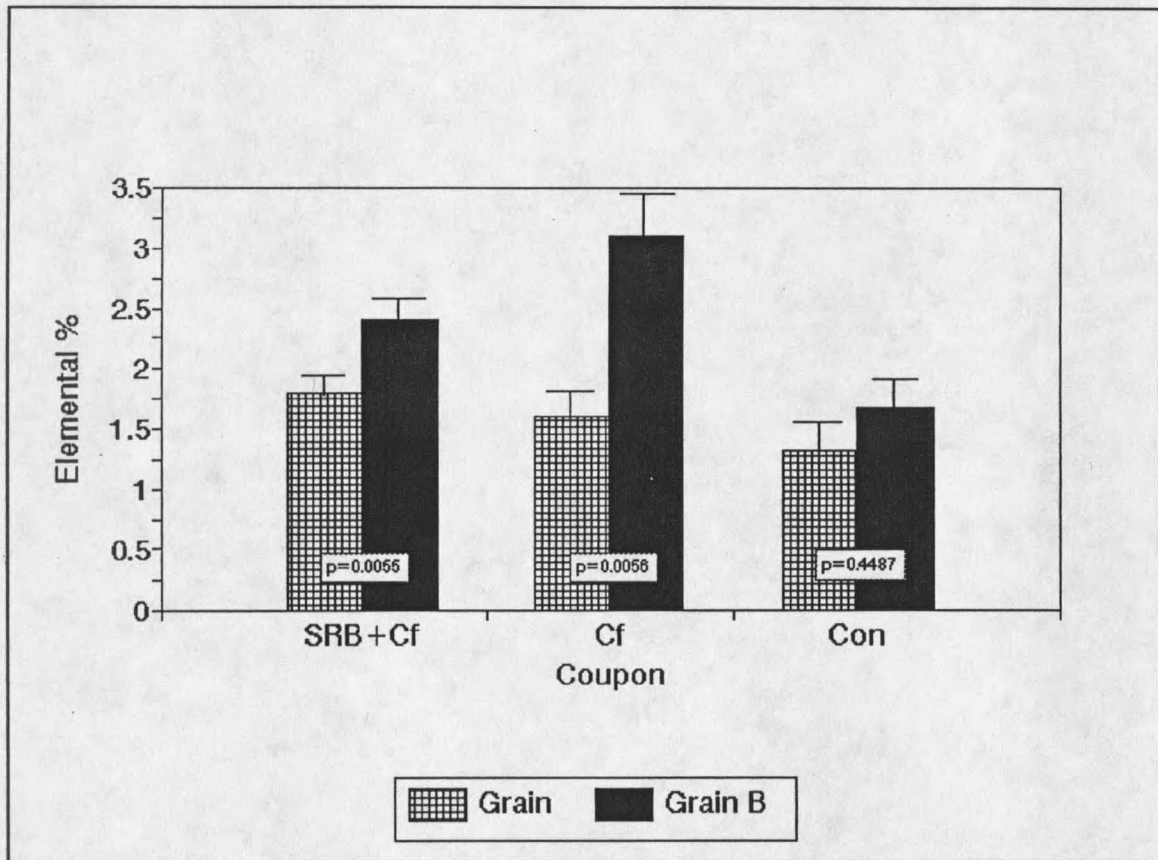


Figure 27. Abundance of nitrogen relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to *C. freundii* (Cf) with or without *D. gigas* (SRB) based on small-spot AES after 60 sec sputter.

coupon, as there were no bacteria present to promote sulfur adsorption. Phosphorus data showed a similar trend (Figure 29) in that higher amounts of phosphorus were noted in the grain boundaries compared to the grains on all coupons. Again, this indicated that the bacteria were not responsible for the phosphorus deposition in the grain boundaries. Therefore, the difference between values when bacteria were present and those obtained from controls indicates contribution of phosphorus and sulfur by the culture medium.

Atomic Force Microscopy

A sample was examined by atomic force microscopy to determine if the features identified as grain boundaries were in fact depressions in the stainless steel surface. Twenty four vertical distance measurements were made between the top of a grain and the bottom of a grain boundary while 12 horizontal measurements were done from each side of a grain boundary (Figure 3). From this data, it was determined that the average depth and width of the grain boundaries on the stainless steel surface was $0.592\ \mu\text{m}$ and $1.554\ \mu\text{m}$, respectively (Figure 30).

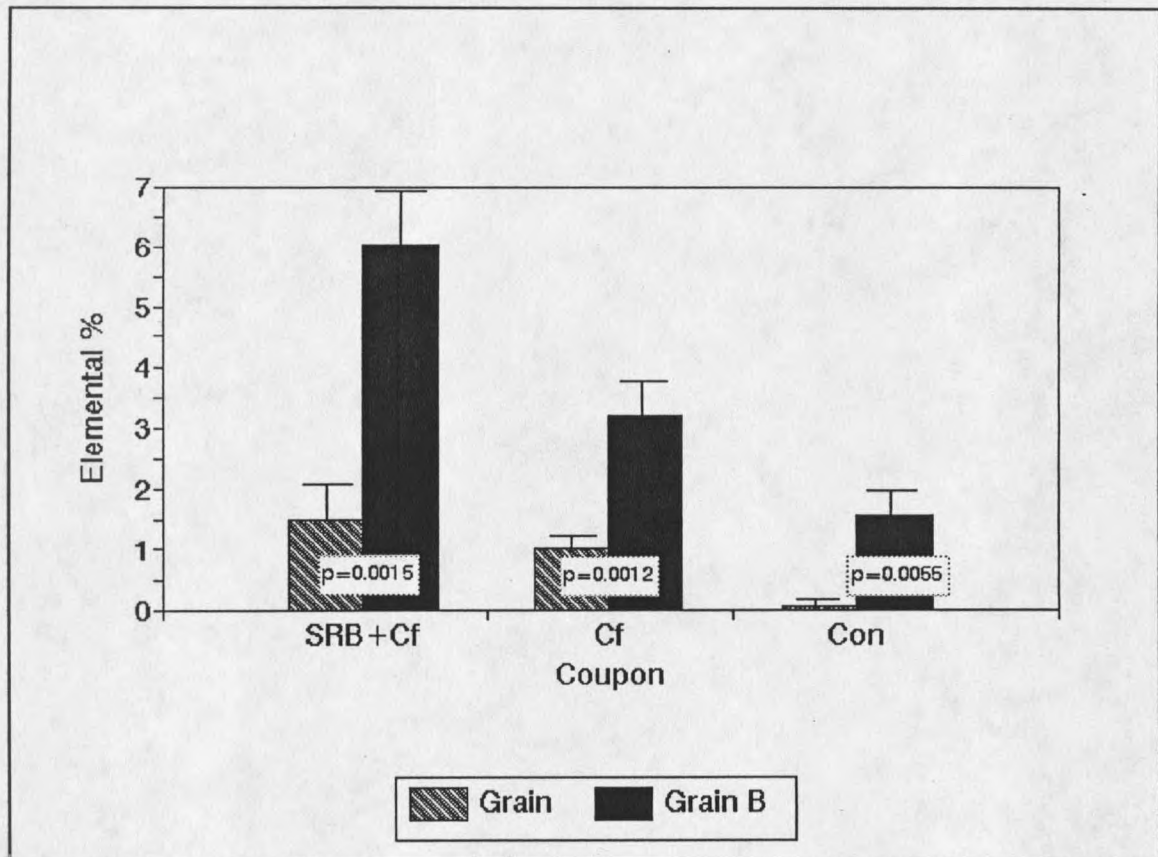


Figure 28. Abundance of sulfur relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to *C. freundii* (Cf) with or without *D. gigas* (SRB) based on small-spot AES after 60 sec sputter time.

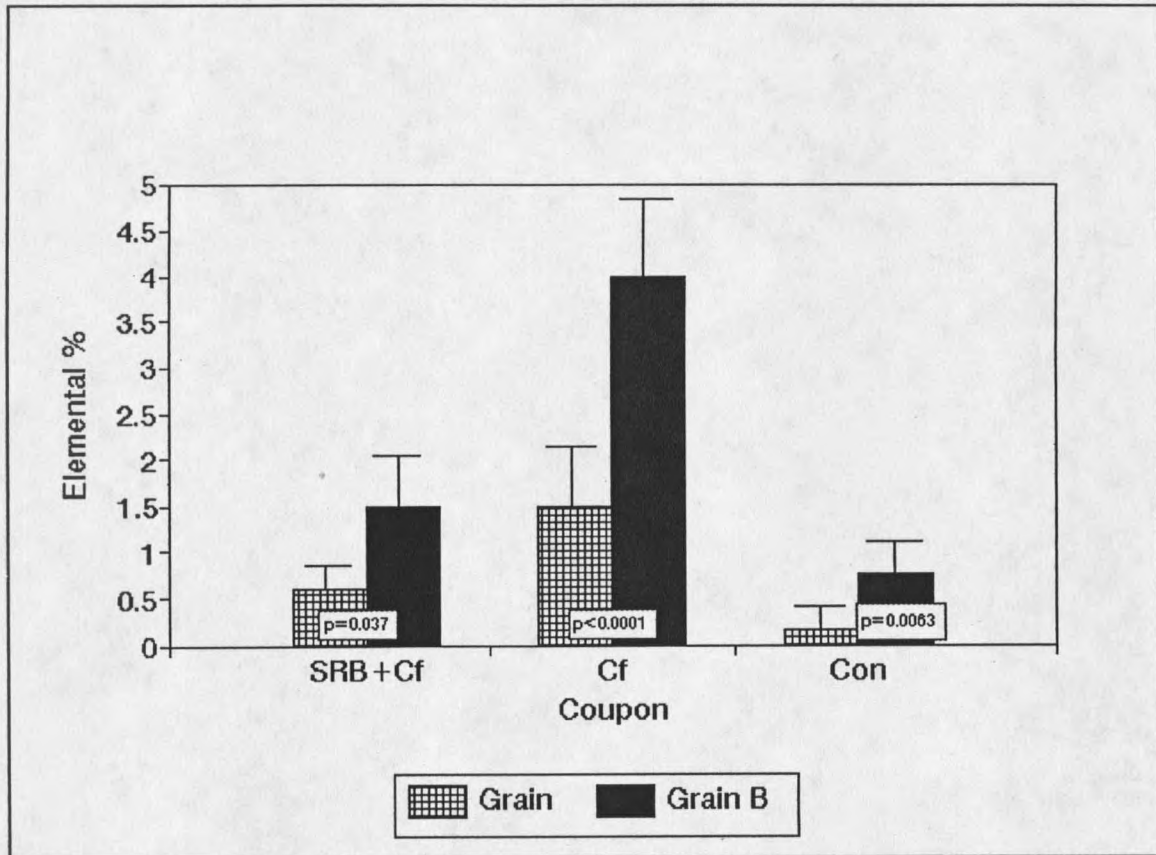


Figure 29. Abundance of phosphorus relative to all other elements in grains and grain boundaries of SS coupons exposed to sterile medium (Con) or to *C. freundii* (Cf) with or without *D. gigas* (SRB) based on small-spot AES after 60 sec sputter time.

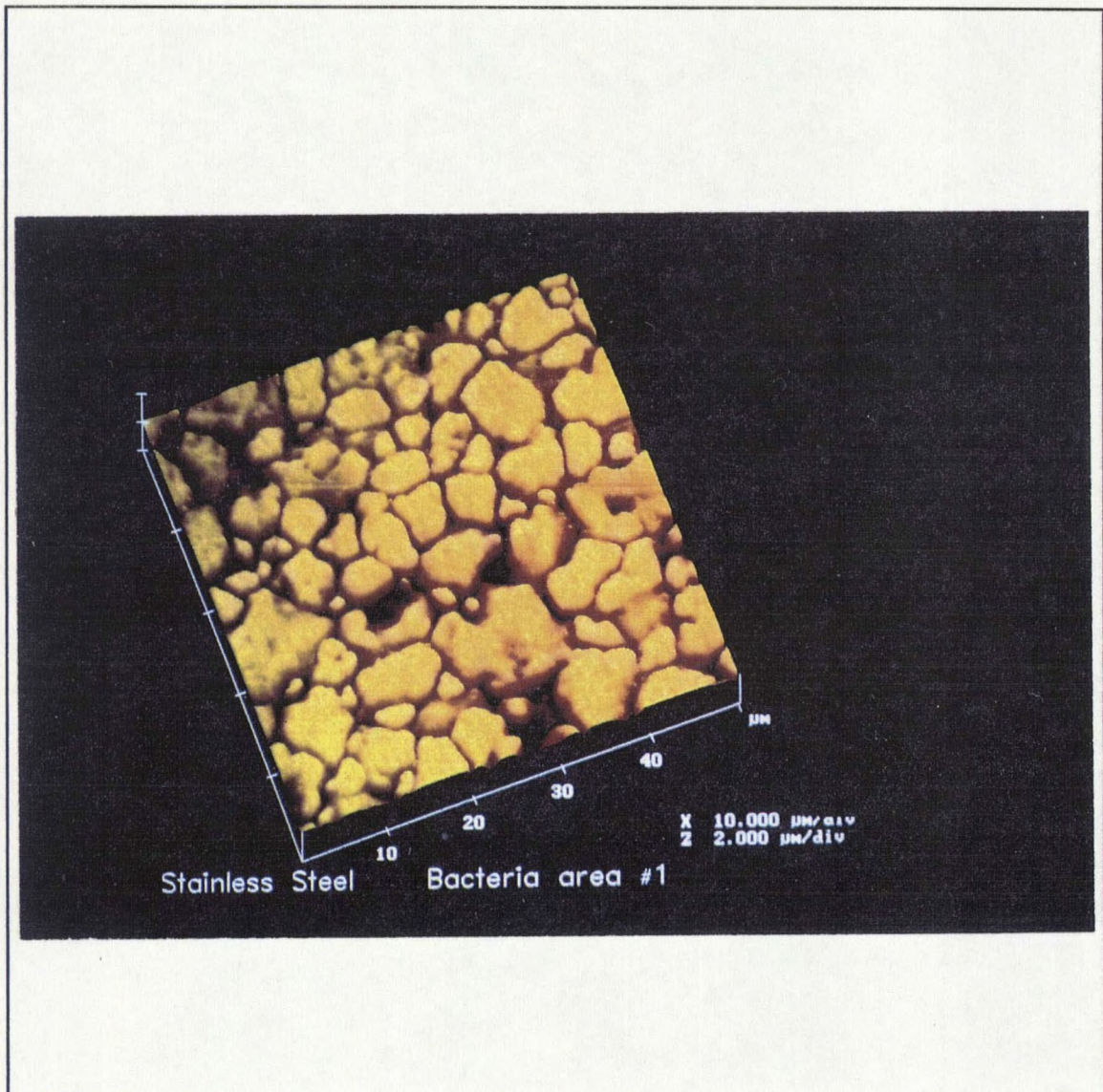


Figure 30. Atomic force micrograph of 316L stainless steel surface, showing three dimensional image of surface.

DISCUSSION

Bacterial Colonization of Grain Boundaries

Colonization of stainless steel by bacteria generally preceeds any adverse effects caused by the bacteria on that surface. In the present study, preferential colonization of grain boundaries by bacteria was demonstrated by a scanning confocal laser microscope in conjunction with image analysis. Statistical analysis of the data indicated a very strong correlation of bacterial partitioning at grain boundaries, with a p value less than 0.0001.

Walsh, *et al*, (76) showed initial attachment to a stainless steel surface to be a random phenomenon, however they went on to say that metallurgical features determine further propagation on the surface and noted preferential attachment of microbes within the grain boundary regions. They also reported high amounts of sulfur and phosphorus within the ferrite phase in austenitic stainless steels after welding and speculate that these could be potential nutrients for microbes. In the present study, small spot Auger analysis indicated that sulfur and phosphorus (Figures 28 and 29) were significantly more abundant in the grain boundaries when compared to the grains on coupons which were only exposed to sterile culture medium. Sulfur and phosphorus in the forms of sulfate and phosphate were present in the culture medium. This would indicate that nutrient deposition

in the forms of sulfate and phosphate occurred within the grain boundaries in the absence of bacteria and is one indication why the bacteria colonized these areas. In environments where sulfur and phosphorus may be limiting, deposition of these nutrients could play a significant role in preferential attachment of bacteria to grain boundaries. The increased amount of phosphorus and sulfur in these areas, whether originating from the stainless steel (76) or the surrounding medium, as shown in this study, is significant and may partially explain why the bacteria preferentially colonize these areas.

Crevices and scratches are often sites of microbial attachment and fouling on stainless steel (56,82). Pedersen (56) reports cells can be shielded from bulk flow on rougher stainless steel surfaces, thus accounting for this phenomenon. In the current study, atomic force microscopy data revealed that the grain boundaries were depressions of an average width and depth of 1.554 μm and 0.592 μm respectively. This is deep and wide enough to harbor bacteria and perhaps protect them from physical stresses such as shear. Tatnall (70) proposes a corrosion mechanism, whereby "free-floating bacteria attach and multiply at low velocity sites such as gasket joints and other crevices". The grain boundaries may create small microzones of low velocity compared to the bulk fluid causing selective attachment and propagation by bacteria within these regions.

Implications of Bacterial Attachment to Grain Boundaries

Once bacteria have colonized grain boundary regions, the integrity and longevity of the stainless steel can be compromised. Grain boundary regions are

inherent weak areas of the stainless steel due to the presence of carbides which cause a localized breakdown in the oxide film (1) and have a thinner chromium oxide surface layer as well as a lower general amount of chromium (up to 40% less) than the adjacent grains in instances where extensive carbide precipitation occurs (7). If there is a localized breakdown in the protective oxide film, localized pitting corrosion can occur (69). If a corrosive bacterial cell preferentially colonized this region and became metabolically active, a number of corrosion-promoting activities can ensue. *C. freundii* is capable of acid production (59). An acid-producing bacterium, like *C. freundii*, could cause localized pitting corrosion, which is the most common type of corrosion to occur on stainless steel (22,27,31,33,61). Although not specifically shown with *C. freundii*, other researchers have observed localized pitting corrosion on stainless steel initiated by iron-oxidizing bacteria, ie *Gallionella*, which cause the formation of corrosive acids (37). This corrosion could initiate in a very localized manner within the grain boundary regions, as was noted by Walsh, *et al* (76).

If bacteria initially colonizing the stainless steel surface were not acid-producers, there could be other means of causing MIC, as follows. These bacteria could form microcolonies, as was observed in Figures 4 and 10, causing a localized depletion in oxygen concentration at the metal surface beneath the microcolony, thus facilitating the formation of corrosive oxygen-differential cells, a proposed mechanism for MIC (14, 73). These microcolonies could also provide niches for other types of organisms, such as SRB, which are most frequently

implicated in localized pitting corrosion of stainless steel (31). SRB located within microcolonies on the stainless steel surface in an aerobic system were shown using a fluorescently-labelled antibody to identify SRB (Figure 4). Interestingly, these thin biofilms in Figure 4 appeared to be slightly more than one monolayer thick, yet SRB were located within these small aggregates. Unfortunately, surface chemical changes under these microcolonies were not measured to determine if these areas were corroding. The idea of SRB existing in thin biofilms in aerobic systems is consistent with studies of Hamilton, 1985, (27) who reports SRB in biofilms thinner than 30 μm and Canfield (11), who report SRB activity within aerobic regions of microbial mats. Although these biofilms observed in this study were very thin on the hand polished stainless steel surface, it seems conceivable that small anaerobic zones were present where the SRB were growing.

Additional phenomena, such as metabolic coexistences may also be occurring. Hamilton (27) and Widdel (77) have hypothesized that SRB could grow in conjunction with facultative anaerobic bacteria. These coexistences could involve interspecies hydrogen transfer or more complex metabolic cross feeding, such as metabolizing short chain fatty acids (77). *C. freundii* was shown to enhance growth of *D. gigas* in anaerobic batch culture (Figure 7) and in the anaerobic reactor system effluent (Figure 8). While this data presumably analyzed suspended growth, on a surface one would expect a metabolic interaction to be more beneficial to organisms in close proximity. Proximity of *D. gigas* to *C. freundii* on stainless steel coupons suspended in anaerobic batch cultures of these

organisms was observed in Figure 10, showing SRB near or in microcolonies of *C. freundii*. These types of associations were seen in all experiments except the anaerobic reactor experiments. Although the explanation of why surface associations were observed in the aerobic reactor and anaerobic batch culture experiments but not in anaerobic reactor experiments remains unclear, one could speculate that in the aerobic system, small anaerobic zones were created by microcolonies of *C. freundii* where the SRB could grow. In the anaerobic batch culture system, as nutrients became exhausted, SRB and *C. freundii* may have migrated to form microcolonies to survive on metabolites exuded by each. In the anaerobic flowing system, with constant nutrient replenishment, this type of close coexistence may not have been necessary.

Bacterial Modification of Grain Boundaries

In the present study, small spot AES was used to determine if surface chemical changes had occurred as a result of bacterial activity within the grain boundaries when compared to grains. The initial data suggested that the stainless steel elements had been removed from the surface, but it was also possible that they may have decreased due to the presence of non-alloy elements. To eliminate the non-alloy element bias, ratios were implemented, which is consistent with other researchers reporting Auger data (47).

In order to calculate ratios, the percentages of the stainless steel elements (chromium, iron, nickel) had to be normalized to a specific element. Nickel was chosen for three main reasons. First, iron was present in the medium, therefore,

iron deposits from the medium could affect the percentage of iron in a particular area. Second, chromium was not a good candidate because of inherent variation in the depth of the chromium oxide passive layer on the surface (6). Nickel appeared to change the least when comparing grains and grain boundaries and was much more homogeneous throughout the chromium-depleted zone than iron or chromium (Table 7). For these reasons, iron and chromium were normalized to nickel, and their respective ratios calculated for each spectrum.

Analysis of the ratios of chromium to nickel and iron to nickel eliminated non-alloy element bias and elucidated possible grain boundary modification by the bacteria and revealed significant decreases in these ratios within the grain boundary regions that were not evidenced on the control. The decrease in ratios indicates one of two possibilities; either iron and chromium were selectively removed in the grain boundary regions as a result of the presence of the bacteria that had preferentially colonized these areas, or the bacteria had selectively enhanced nickel concentration within the grain boundaries. In order for the second scenario to occur, nickel would have to be selectively removed from another portion of the stainless steel. This would have caused the relative amounts of chromium and iron to increase in these areas where nickel was removed. If this had happened, the raw percentage AES data would have indicated no difference in the means of chromium and iron as there would have been some areas that contained higher amounts, and other areas that contained lower amounts, with the overall difference being negligible. This was not observed,

therefore the second scenario seems doubtful.

The first possibility of chromium and iron preferentially removed over nickel seems much more probable. AES raw elemental percentage data indicated possible changes in chromium and iron, rather than nickel. These changes could not have been an artifact of iron deposition from the medium and chromium heterogeneity on the surface as the differences in iron and/or chromium were not seen on the coupons exposed to sterile culture medium. Also, nickel is generally thought to be a corrosion-resistant material. Higher composition nickel alloys (40% nickel as in Alloy 825) are more corrosion resistant than austenitic stainless steels (10% nickel) and are being recommended for use when corrosion resistant materials are needed (Gustavo Cragnolino, personal communication).

Chromium and iron depletion can have significant effects with respect to susceptibility to localized corrosion initiation. Grain boundary chromium depletion on stainless steel has been suspected for many years, but until recent analytical techniques were perfected, little additional information was available (6). Advani, *et al*, (1) studied chromium depletion development within grain boundaries on 316 stainless steel using an indirect electrochemical potentiokinetic reactivation (EPR) method. They found that grain boundary chromium depletion (GBCD) causes localized breakdown in the chromium oxide passive layer and leaves these regions susceptible to intergranular corrosion and stress corrosion cracking. Lumsden and Stocker (44) used AES to study GB CD on 304 stainless steel and found these depleted areas to be susceptible to intergranular corrosion. Intergranular

corrosion caused by bacteria was thought to be the cause of the localized pitting corrosion reported by Deshmukh, *et al* (16). Iron depletion in any iron-based alloy can have detrimental effects to material stability and longevity.

Figure 31 is a conceptual model of the selective removal of iron and chromium relative to nickel within a grain boundary covered by a patchy biofilm. As previously shown in Figure 15, and with the AES data, iron and chromium are not in significantly different concentrations when comparing grain boundaries and grains prior to bacterial attack. However, as bacteria colonize these areas, the aggressive bacterial actions permit the preferential dissolution of chromium and iron, presumably by a localized breakdown in the chromium oxide layer followed by dissolution of iron within the grain boundary area.

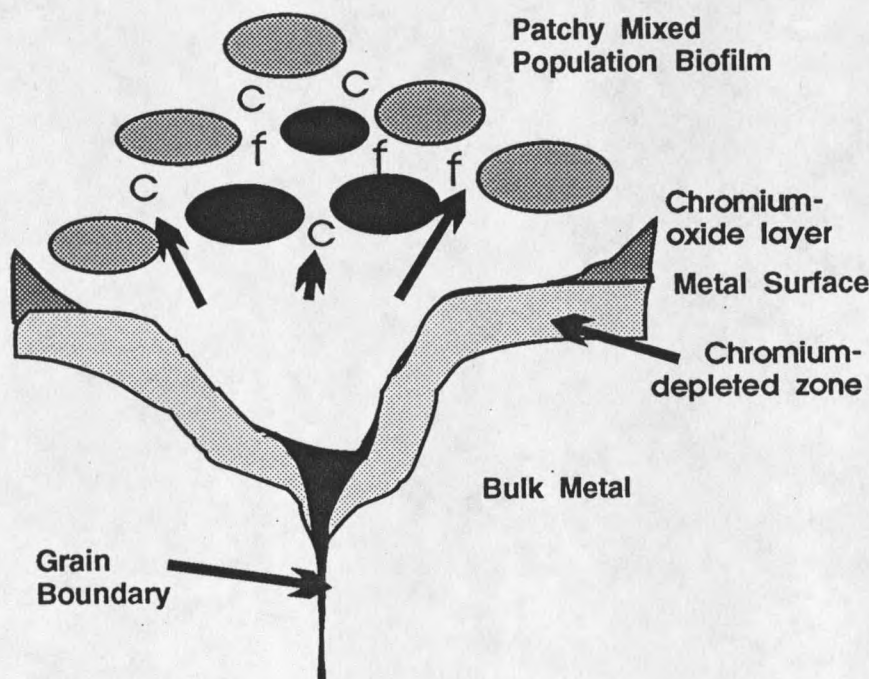


Figure 31. Conceptual model of initial corrosion events whereby a patchy mixed culture biofilm is covering a grain boundary and causing the selective removal of chromium (c) and iron (f).

Potential Prevention of MIC on Stainless Steel

This study shows that bacterial colonization occurs in a preferential manner on grain boundaries of 316L stainless steel. Preferential colonization leads to chromium and iron depletion in these sensitive areas. A remedy to protect or shield these sensitive areas may be to look for surfaces that are more homogeneous. One such surface is an electropolished surface. Electropolishing causes a thick (up to 50 Å) chromium oxide layer to form on a stainless steel surface (Tim Daly, personal communication). Walsh, *et al*, (76) showed initial attachment to a stainless steel surface to be a random phenomenon, however they went on to say that metallurgical features determine further propagation on the surface. If these metallurgical features, such as grain boundaries, could be shielded by processes like electropolishing, this may cause a more uniform propagation of bacteria on the surface, rather than a selective attachment and subsequent attack.

Conclusions

Preferential colonization of grain boundaries of 316L stainless steel by *C. freundii* was observed using a novel combination of scanning confocal laser microscopy and image analysis. Results indicate nutrient availability and protection from physical forces as possible reasons for this phenomenon.

The AES data indicate that *C. freundii* caused the selective removal of chromium in the grain boundaries, while the combination of *C. freundii* and *D. gigas* caused the selective removal of iron in the grain boundaries. Evidence

indicates that the longer the coupons are in contact with the bacteria, the greater the depletion. This study shows quantitatively that grain boundaries are preferentially attacked by bacteria. Although it is impossible to eliminate grain boundaries as they are an inherent feature of any alloy, some techniques like electropolishing may slow the MIC process on stainless steel and in doing so, increase system life and material longevity.

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APPENDIX

Passivation Procedure

Hutner Salts Formulation

1. Cleaning-Passivation with Nitric Acid Solution

50% nitric acid at 71°C for 30 minutes

2. Modified Hutner Salts solution

Concentrated base (add to 1000 ml distilled water):

Nitrilotriacetic acid	10.0 g
MgSO ₄	14.45 g
CaCl ₂ ·2H ₂ O	3.335 g
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	9.25 mg
FeSO ₄ ·H ₂ O	99.0 mg
Metals "44"	50.0 ml

Metals "44" contains per 100 ml:

Disodium Ethylenediaminetetraacetate	250.0 mg
ZnSO ₄ ·7H ₂ O	1095.0 mg
FeSO ₄ ·7H ₂ O	500.0 mg
MnSO ₄ ·H ₂ O	154.0 mg
CuSO ₄ ·5H ₂ O	39.2 mg
Co(NO ₂) ₂ ·6H ₂ O	24.8 mg
Na ₂ B ₄ O ₇ ·10H ₂ O	17.7 mg

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