MICROBIOLOGICALLY INFLUENCED CORROSION OF STEEL COUPONS IN STIMULATED SYSTEMS: EFFECTS OF ADDITIONAL NITRATE SOURCES

By

MRS CHARLENE PILLAY

Submitted in fulfilment of the academic requirements for the degree of Doctorate in Microbiology in the School of Life Sciences, University of KwaZulu-Natal, Durban

November 2012

ABSTRACT

Microbiologically Influenced Corrosion (MIC) is a process influenced by microbial presence and their metabolic activities. This study examined the microbial effects on metal corrosion under different environmental conditions with nutrient supplements. Experiments were conducted by inserting stainless steel 304 and mild steel coupons (2.5 x 2.5 cm^2) into loam soil and a simulated seawater/sediment system with various nutrient conditions (sterilized, without supplement, 5 mM $NaNO_3$ or NH_4NO_3). Two mild and stainless steel coupons were removed monthly and the corrosion rate was evaluated based on the weight loss. Bacterial populations were enumerated using the most probable number (MPN) technique. The presence and adhesion of microbes on mild steel coupons were examined using Scanning Electron Microscopy (SEM). The extent of the corrosion process on the surface of the metal coupons were visualized by using the Stereo Microscope. The elemental composition of the corrosion products formed on the coupon surface were determined by Energy Dispersive X-Ray analyses. Isolation and identification of aerobic microorganisms were conducted and examined for its potential in either accelerating or inhibiting corrosion. The bacterial populations present on the mild steel surface were analyzed by fluorescent *in situ* hybridization. Denaturing gradient gel electrophoresis (DGGE) analyses of PCR-amplified 16S rDNA fragments were conducted to determine the microbial community complexity of the biofilm. Greater weight losses of mild steel in loam soil and the seawater/sediment system with $NaNO_3$ (48.86 mg/g and 19.96 mg/g of weight loss, respectively after 20 weeks) were observed with total heterotrophic bacterial population presented (106.695 MPN/ml and 0.11187 MPN/ml respectively) compared to the autoclaved control (7.17845 mg/g and 0.12082 mg/g of weight loss respectively). Supplementation of 5 mM NH_4NO_3 increased the total heterotrophic bacterial population and resulted in a decrease in weight loss measurements on the stainless steel coupons (211.4 MPN/ml with a 0.01 mg/g weight loss) after 20 weeks compared to the non-autoclaved loam soil and loam soil supplemented with $NaNO_3$ (139.2 MPN/ml and 134.9 MPN/ml respectively with no weight loss). SEM images of the mild steel coupons confirmed the presence and adherence of bacteria on the metal surface. Stereo microscopic images displayed reddish-brown deposits and pitting on the coupon surface. Isolation, identification and sequence analysis revealed that most microorganisms were the *Bacillus* species. This group of microorganisms are iron-oxidizing bacteria that could also promote the corrosion process. After 20 weeks of incubation, the total SRB cell counts were lower in samples supplemented with $NaNO_3$ in both loam soil and the seawater/sediment system. This

study also indicated that the isolated aerobic microorganisms do play a role in the corrosion process in both stainless and mild steel. DGGE analysis revealed microbial diversity in the corrosion products especially those affiliated to the bacterial phyla Firmicutes and Gamma-Proteobacteria. Fluorescent *in situ* hybridization analysis allowed for an overall estimation of Eubacteria and sulphate-reducing bacteria present in the biofilm formed on the surface of mild steel. The current study indicates that the addition of nitrates did not significantly reduce the rates of corrosion of both mild and stainless steel. However, it does seem that environmental conditions did pose as an important factor in the corrosion process. Therefore, further studies need to be implemented to analyze the environmental type, microbial composition and optimization of the concentration of nitrates for possible mitigation of metal corrosion. To optimize MIC prevention and control, collaboration between engineers and microbiologists proves advantageous to develop an environmentally sound and potentially cost-effective approach to control corrosion.

Keywords: Microbiologically Influenced Corrosion, nutrient supplements, bacterial population, weight loss

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville, from July 2007 to November 2012, under the supervision of Professor Johnson Lin.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 1 - PLAGIARISM

I, Mrs Charlene Pillay, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

……

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

Publication 2

Publication 3

etc.

Signed:

ACKNOWLEDGEMENTS

- My promoter Prof. J. Lin for his motivation, supervision and support during this study.
- The National Research Foundation for financial assistance.
- Ernst and Ethel Trust Fund.
- The staff and postgraduate students of the Microbiology Department (UKZN, Westville Campus) for their support and much appreciated assistance.
- Mr Charles Baah (Engineering Department, Howard College).
- The Corrosion Institute of Southern Africa for the Ivan Oligvie Award.
- My parents, Koos and Romilla Singh for their unconditional love and support throughout my study.
- My husband, Malcolm Pillay for his undying love, motivation, support and tolerance through my toughest times.
- The rest of my family for their encouragement and love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

In natural and man-made environments, corrosion occurs when materials made of pure metals and/or their mixtures (alloys) undergo a chemical change (Beech and Gaylarde, 1999; Beech, 2003). Corrosion is defined as an electrochemical process involving the transfer of electrons in the presence of an electrolyte through a series of oxidation and reduction reactions (Beech and Gaylarde, 1999; Beech, 2003; Beech and Sunner, 2004). Corrosion can lead to potentially hazardous system malfunctions as well as increases in the damage and repair costs (Lutterbach and de Franca, 1997; Chen *et al*., 2007; Akpabio *et al*., 2011). Some readily noticeable effects of corrosion include changes in drinking water taste, odour and appearance. Other results of corrosion include pinhole leaks, leaching of metals into the drinking water, gastrointestinal distress and even catastrophic pipe failures. Ingestion of these metals above their maximum contaminant levels can affect consumer health and have been shown to cause gastrointestinal illnesses (Beech and Gaylarde, 1999). In South Africa, the direct cost of biocorrosion has been estimated at around 400 million rands per annum and at 16 to 17 billion US dollars per annum in the USA (Coester and Cloete, 2005). The costs associated with repairs and down time are hundreds of billions of rands annually (Beech and Gaylarde, 1999). The direct cost of corrosion to the South African economy was estimated at 154 billion rands per annum (McEwan, 2005).

Biocorrosion is directly related to the presence and adherence of microorganisms on different industrial surfaces, resulting in metal damage (Guiamet and Gomez de Saravia, 2005). The deterioration of metal due to microbial activities that modify the local chemistry at a metal interface, such that localized corrosion is promoted or inhibited, is termed Microbiologically Influenced Corrosion [MIC] (Angell, 1999; Beech and Sunner, 2004; Chen *et al*., 2007; Landoulsi *et al.*, 2008). Bacterial cells can encase themselves in a hydrated matrix of polysaccharides and protein and form a slimy layer known as a biofilm (Guiamet and Gomez de Saravia, 2005). Formation of a biofilm creates a microenvironment that is dramatically different from the bulk surrounding (Akpabio *et al*., 2011). The biofilm can be considered as a gel containing 95% or more water, microbial metabolic products including enzymes, extracellular polymeric substances (EPS),

organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulphide and inorganic detritus (Guiamet and Gomez de Saravia, 2005; Videla and Herrera, 2005; Perez *et al*., 2007; Landoulsi *et al*., 2008). Biocorrosion constitutes approximately 10% of all metallic corrosion.

Microorganisms implicated in biocorrosion of metals such as iron, copper and aluminium and their alloys are physiologically diverse (Beech and Gaylarde, 1999). Their ability to influence biocorrosion of many metals otherwise considered corrosion resistant in a variety of environments, makes microorganisms a real threat to the stability of such metals. The main types of bacteria associated with metal corrosion are sulphate-reducing bacteria (SRB), sulphur-oxidising bacteria, iron-oxidising/reducing bacteria, manganese-oxidising bacteria, and bacteria secreting organic acids and exopolymers (Beech and Gaylarde, 1999; Lee *et al.,* 2006). These microorganisms can coexist in naturally occurring biofilms often forming synergistic communities that are able to affect electrochemical processes through co-operative metabolism not seen in the individual species (Beech and Gaylarde, 1999).

One of the classic concepts for maintaining an industrial system free of the deleterious effects of MIC is "to keep the system clean". Prevention and treatment of biocorrosion should be mainly based on avoiding or minimizing the development of biofilms. There are several general methods such as physical or chemical treatments that can be used to remove corrosion products from an industrial system. Physical methods include flushing and the use of abrasive and non-abrasive sponge balls to remove any potential factors involved in metal corrosion acceleration. The most common chemical method is the use of biocides that are single compounds (either oxidizing or non-oxidizing agents) capable of killing microorganisms or inhibiting microbial growth (Videla and Herrera, 2005). The effectiveness of a biocide depends on the nature of microorganisms to be eliminated and the operating conditions of the system to be treated (Videla, 2002). Chlorine, ozone and bromine are three typical oxidizing agents used in industries. Typical non-oxidizing biocides are formaldehyde, glutaraldehyde, isothiazolones and quaternary ammonia compounds (Jayaraman *et al.,* 1999a).

The lack of efficacy of biocides against sessile organisms is probably due to the inability of the chemical to penetrate the biofilm, in addition to physiological differences between sessile and planktonic cells. Biocides are often inherently toxic and are frequently difficult to degrade by being

persistent in the natural environment or able to accumulate in a variety of matrices and often causing contamination of areas distant from the site of treatment. Thus, biocides may have a negative impact on the environment if they are applied without a proper environmental risk assessment. Environmental concern has led to legislation which encourages the replacement of toxic biocides, widely used in the past with more readily degradable antimicrobial chemicals that are compatible with operating systems and less toxic to the environment (Guiamet and Gomez de Saravia, 2005; Videla and Herrera, 2005).

Another method for the mitigation and control of sulphides that occurs as a result of SRB activities, involves the addition of nutrients (Gevertz *et al*., 2000; Videla and Herrera, 2005). The influx of sulphate can stimulate indigenous SRBs, which metabolize the sulphate into hydrogen sulphide gas. The hydrogen sulphide then reacts with metallic compounds such as iron to form iron sulphide, which appears as a black scale. The introduction of nutrients stimulates the activity of indigenous nitrate-reducing, sulphide-oxidising bacteria to lower sulphide concentrations, as well as stimulating heterotrophic denitrifiers that can outcompete SRB for energy sources and common electron donors (e.g. organic acids), thereby allowing nitrate-reducing bacteria to exclude sulphate-reducing bacteria by a strategy known as "biocompetitive exclusion" (Gevertz *et al*., 2000). The biocompetitive exclusion process provides several positive benefits:

- simple treatment and sustainable results;
- the reduction of hydrogen sulpide (H_2S) gas and iron sulphide (FeS) deposits that can lead to significant production problems and safety hazards;
- reduced corrosion and increased life of production equipment; and
- reduced cost compared to conventional treatments with biocides, corrosion inhibitors and hydrochloric acid (Gevertz *et al*., 2000).

Metal corrosion are of great concern to many industries in South Africa and world-wide. In particular, these industries are seriously affected by the metabolic activities of microorganisms. The negative effect of microbial corrosion of metals on the environment and the economy is of paramount importance. It is crucial to understand the process of metal corrosion especially MIC, and to develop some technology to control its negative impact. At the same time, the developed strategies have to avoid any negative impacts in our living environment. Therefore, this study is

aimed at evaluating the process of corrosion of mild and stainless steel with the addition of nitrates under different environmental conditions.

1.2 LITERATURE REVIEW

1.2.1 METAL CORROSION

Corrosion is a naturally occurring process in which materials fabricated of pure metals and/or their mixtures (alloys) undergo chemical oxidation from ground state to an ionized species (Beech, 2003). This process proceeds through a series of oxidation and reduction reactions of chemical species in direct contact with, or in close proximity to, the metallic surface (Beech and Sunner, 2004). Complex deposits of microbial cells, extracellular polymers, and organic and inorganic debris that accumulate on the metal surface accelerate corrosion by changing the electrochemical behaviour of the metal (Dickinson *et al.*, 1997).

The electrochemical process (Figure 1.1) has three essential constituents: an anode, a cathode and an electrically conducting solution. Corrosion occurs when a current leaves a structure at the anode site, passes through an electrolyte and reenters the structure at the cathode site, also known as oxidation. The anode is the site at which the metal is corroded; the electrolyte solution is the corrosion medium; and the cathode forms the other electrode of the cell and is not consumed in the corrosion process (Rothwell, 1979; Al-Faiz and Mezher, 2012). The adherence of oxidation products to the surface forms one or more layers that serve as a diffusion barrier to the reactants. These layers, depending on their chemistry and morphology, may act as a protective barrier against further metal deterioration. Changes in the environmental conditions can affect the stability of the protective layers and therefore the overall susceptibility of the material to corrosion (Beech, 2003). The potential range of film presence and stability is recognized as the passive region and extends from the potential of oxide formation to the pitting or breakdown potential at which the rupture of the oxide film determines the localized corrosion failure (Leda *et al*., 2006).

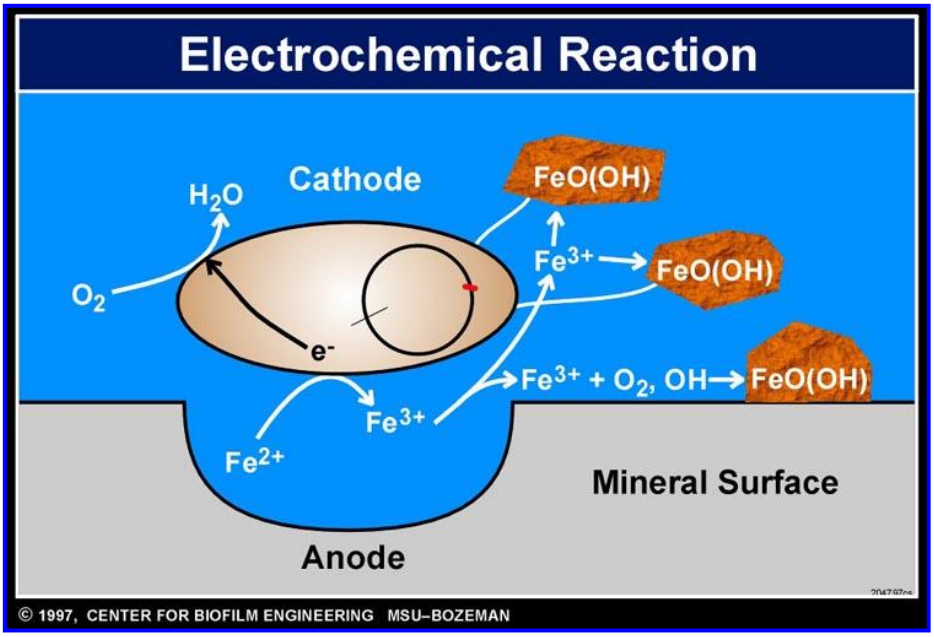

Figure 1.1: Schematic diagram of the electrochemical reaction taking place at the mineral surface (Geesey and Phinney, 1997)

1.2.1.1 TYPES OF METAL CORROSION

Corrosion comes in many different forms and can be classified by the cause of the chemical deterioration of a metal such as general/uniform corrosion, pitting, galvanic and crevice corrosion as seen in Table 1.1 (Melchers, 2003a). General (uniform) corrosion occurs uniformly over a wide area of the metal surface producing a general thinning of the metal leading to eventual failure. The rate of general corrosion is usually relatively predictable. It is often combined with high-velocity fluid erosion, with or without abrasives. Rusting steel in the atmosphere and the corrosion of copper alloys in seawater are common examples where uniform corrosion is encountered (Colavita, 2000).

Pitting corrosion is evident on metal surfaces where there is no presence of uniform corrosion and usually highly localized. Pitting often proceeds rapidly, is accelerated by the presence of chlorides and is particularly common at the base of breaks in coatings. Pitting corrosion takes advantage of the different metallurgical phases present on the surface of most common alloys. This type of

corrosion if is often combined with stagnant fluid or in areas with low fluid velocity (Colavita, 2000; Gurappa, 2002).

A potential difference usually exists between two dissimilar metals when immersed in a corrosive solution (Fontana and Greene, 1967). When dissimilar metals are connected in the presence of an electrolyte, a galvanic corrosion reaction occurs. Electrolytes are electrically conductive solutions, such as seawater, and are the most common source of chloride contamination (Azumi *et al*., 2000). Corrosion of the less corrosion-resistant metal is usually increased and attack of the more resistant material is decreased, as compared with the behavior of these metals when they are not in contact. The less resistant metal becomes anodic and the most resistant metal cathodic (Fontana and Greene, 1967). The anodic metal develops deep pits and grooves in the surface (Azumi *et al*., 2000).

Crevice corrosion occurs in sheltered, localized areas such as crevices, joints, bolted and threaded parts; and under existing corrosion deposits. It is the result of a concentration of salts, acids and moisture which results in the formation of an occluded corrosion cell in such sheltered areas. A small anode is created in the crevice with the remainder of the body acting as a large cathode so corrosion at the crevice is highly accelerated as well as concentrated. Crevice corrosion creates pits similar to pitting corrosion. All stainless steel grades are considered susceptible, but their resistance varies. Their resistance to attack is largely a measure of their chromium, molybdenum and nitrogen content (Laitinen, 2002).

While the instantaneous corrosion rate may be used as a guide to estimate the probable loss of steel, the main concern remains over the accuracy of monitoring techniques and the reliability of such estimates. The rate of corrosion in steel normally fluctuates with time. The degree of fluctuation can depend on the mechanism of corrosion occurring. Metal corrosion is a dynamic process and subject to random variations even under constant conditions. The local environment is very sensitive to the microclimate in which they exist, in particular the moisture content (Law *et al*., 2004).

Table 1.1: Types of metal corrosion (Melchers, 2003a)

TYPES OF CORROSION	MATERIALS	FEATURE	PROBLEM AREAS	EFFECT
General (or "uniform")	Mild steels, high tensile steels	Roughly uniform over extended areas	Plating	Reduction of plate thickness, structural capacity
Pitting	Limited for mild steels, mainly high tensile and stainless steels	Highly localized penetration, often with clusters of pits	Plating, local details	Local reduction in thickness and stress intensification, possibly leakage
Galvanic	Dissimilar metals in contact	Localized	Different metals in contact, variation in metal composition	Localized severe material loss
Crevice	Mainly stainless and some high tensile steels	Highly localized elongated penetration	At connections, under washer, etc.	Localized stress intensification, local failure

1.2.1.2 FACTORS AFFECTING CORROSION IN VARIOUS ENVIRONMENTS

1.2.1.2.1 SOIL ENVIRONMENTS

Soils are known to promote corrosion of buried metallic structures such as pipe lines for water, gas, oil, transmission lines, tanks, power cables, etc. The presence of moisture, air and electrolytes are factors promoting corrosion in soils. When metals are laid out in soils, corrosion is caused by the formation of differential aeration and concentration due to soils of different compositions and textures present along the metal structures (Dayal *et al*., 1988; Lopez *et al*., 2006). Normally in the absence of air, there should be no corrosion under neutral conditions as the oxidant-cum-cathodic depolarizer oxygen is absent (Mirgane and Ramdas, 2009). However, extensive corrosion has been reported under such conditions due to the activities of anaerobic SRB (Dayal *et al*., 1988). If atmospheric oxygen cannot penetrate the soil, in the case of clay or peat soils, the hydrogen produced as a consequence of the cathodic reaction in the underground structure can be eliminated (oxidized) due to microbial factors (AlHazzaa, 2007). This process is the result of the metabolic activity of bacteria in anaerobic conditions. Microbial corrosion is due to the presence of a biofilm on the metal surface, located between the metallic substratum and the surface liquid. Such biofilms influence corrosion since they bring about chemical changes in the proximity of a metal surface. It

is also known that in biotic anaerobic systems, sulphate bacteria can considerably increase corrosion due to the production of hydrogen sulphide (Lopez *et al.*, 2006).

If other soil factors, viz. moisture, temperature, texture and electrolyte content are constant, there is a correlation between the soil resistivity and the rate of corrosion. Soil resistivity is a general indicator of the environment's ability to support the electrochemical reactions that are part of the corrosion mechanism (the lower the resistivity, the higher the corrosion) (Dayal *et al.*, 1988). In addition, the resistivity depends upon soil structure features such as porosity, permeability, moisture level, ion content and acidity (Lopes *et al*., 2006). The soluble salt content of the soils is important in corrosion of sub-soil structures. Besides forming electrochemical cells and supporting corrosion, these salts serve as a source of food for soil microbes and enhance their metabolic activities. The physical and mechanical properties of soils are relevant to its corrosivity as these factors hold a key to soil resistivity, water-holding capacity and rate of aeration. Soils vary widely in their physical, chemical and electrical properties (Dayal *et al.*, 1988).

Buried pipelines that span long distances are known to pass through varied soil textures, depths and in some places insidious ions that add to the corrosivity of the soil (Dayal *et al*., 1988; Oguzie *et al*., 2004; Lopez *et al*., 2006). In practice, corrosion susceptibility in soil determines the service life of the buried metallic structure. However, the service life as well as corrosion in soil also depends on the metal thickness, the area exposed and repairs and/or the maintenance technique employed (Oguzie *et al*., 2004). Thus, the corrosion tendency in soil varies from one location to another, due to the nature of the soil chemistry which in addition depends on aeration, dissolved salts and resistivity (Dayal *et al*., 1988; Oguzie *et al*., 2004).

1.2.1.2.2 MARINE ENVIRONMENTS

Seawater is a complex chemical system affected by various factors (Moller *et al*., 2006). It is one of the most corroded and abundant naturally occurring electrolytes (Johnsirani *et al*., 2012). Predicting the rate of corrosion of steel in a marine environment is a challenge due to the wide variation of parameters controlling the rate (Paul, 2012). The factors that determine the type of corrosion attack on metal and its rate can be classified as chemical, physical and biological (Farro *et al*., 2009). The degree of corrosion severity in marine environments is dependent on many parameters including temperature, dissolved oxygen content, salinity, water chemistry, pH, biofouling, time-of-wetness,

high atmospheric contaminants (i.e. chlorides and SO_2), pollution and fluid velocity as seen in Table 1.2 (Boyd and Fink, 1978; Davis, 1987; Melchers, 2003c; Jaen *et al*., 2012). The severity of corrosion in a marine environment is related to the time of wetness during which time, electrochemical processes and corrosion takes place. There is a direct relationship between the salt content and corrosion rate (Griffin, 1987; Johnsirani *et al*., 2012).

The marine environment includes several zones, to which the metal structure can be exposed and these determine the degree of its corrosion and the specific corrosion attack. The splash and tidal zones are the areas where the metal is more affected by the corrosion process due to the formation of differential aeration cells (Farro *et al*., 2009). The splash/spray zone has been characterized as an aerated seawater environment where exposed metals are almost continually wet and biofouling organisms do not attach (Compton, 1971; Boyd and Fink, 1978). The tidal zone is an environment where metals are alternately submerged in seawater and exposed to the splash/spray zone as the tide fluctuates. Metals are influenced by tidal flow, where increased movement due to tidal action may cause increased corrosion (Boyd and Fink, 1978). The rate of immersion corrosion is known to increase with water velocity (Melchers and Jeffrey, 2004). The shallow ocean environment is typically characterized by well-aerated water combined with both plant and animal marine biofouling organisms. As in the tidal zone, biofouling organisms can either protect the metal surface from attack or can accelerate corrosion at the attachment site (Compton, 1971; Boyd and Fink, 1978).

The seawater temperature is dependent on seasonal and geographic factors. As a general rule, corrosion reaction rates in seawater increase as the temperature increases (Boyd and Fink, 1978; Davis, 1987). The dissolved oxygen is the most important factor in the corrosion process, and its transport by diffusion, towards the surface of the metal is the decisive stage of the corrosion kinetics (Farro *et al*., 2009). The solubility of oxygen decreases as the temperature increases (Boyd and Fink, 1978; Davis, 1987). The oxygen levels increase due to photosynthesis by marine plants and by wave action, while the oxygen levels decrease due to the demand for oxygen created from organic matter decomposition (Dexter and Culberson, 1980; Farro *et al*., 2009). The major chemical constituents of seawater are consistent worldwide. In seawater, pH is remarkably uniform, mainly due to the buffering capacity of seawater. Typically, the pH has a small daily fluctuation in the range of 8.0–8.2. Although changes in pH do not alter the corrosion behaviour of most metals, the variations can influence the formation of protective calcareous scales, which may affect corrosion

rates. However, seawater marine conditions, in particular, are known to be highly corrosive for most common steels (Moller *et al*., 2006). Salinity is closely tied to the calcium and magnesium carbonate balance (hardness) in the seawater and to pH (Lebedev and Derbyshev, 1978; Melchers, 2006). pH has a variable effect on corrosion. Corrosion rate is generally higher at a lower pH due to acidic corrosion, while at a pH of 8.5 to 12, it decreases due to the formation of a passive layer (Paul, 2012). A low pH of seawater could be due to the presence of atmospheric pollutants, found at highly polluted coastal locations or of high industrial activity (Farro *et al*., 2009).At a higher pH, corrosion is known to be severe (Paul, 2012). Changes in pH and in water hardness are responsible for the changes in corrosivity (Lebedev and Derbyshev, 1978; Melchers, 2006). In general, when either the temperature or salinity increases, the dissolved oxygen content in seawater decreases (Dexter and Culberson, 1980; Davis, 1987). Both temperature and dissolved oxygen have a strong effect of increasing the corrosion as the concentration of dissolved oxygen increases the rate of cathodic reaction of water reduction (Paul, 2012).

Calcium and magnesium carbonate deposits form part of the corrosion product layer and play a role in regulating the supply of oxygen to the corrosion interface (Melchers and Jeffrey, 2005; Melchers, 2006a). Sulphate ion helps the formation of corrosion resistant deposit with calcium and magnesium ions, but at a lower pH, this deposit goes into solution and the corrosion rate is enhanced (Paul, 2012). Increased pH of the seawater will increase calcite deposition owing to the changed solubility. This provides greater protection against aerobic corrosion, through limiting oxygen diffusion (Melchers and Jeffrey, 2005; Melchers, 2006a; Farro *et al*., 2009). In coastal regions, many coastal infrastructures include facilities constructed from structural-grade steels, e.g. sheet-piling, bridges, jetties, pipelines, off-shore structures, and ships. The seawater here might change in composition due to river-flows or storm-water runoff. Water pollution of coastal waters, harbors, and river estuaries is often associated with excessive corrosion (Melchers and Jeffrey, 2005; Melchers, 2006a). However, the reasons for this kind of corrosion are seldom reported in sufficient detail for correlation with corrosion studies (Melchers, 2006a).

Table 1.2: Factors which may influence marine immersion corrosion (Melchers, 2003a)

FACTOR	EFFECT ON INITIAL CORROSION RATE	EFFECT ON STEADY STATE CORROSION RATE	INFLUENCED BY
BIOLOGICAL			
Bacterial	None	Reduces and probably controls rate	Temperature of seawater
Biomass/plant life	None	Varies	NaCl concentration
Animal life	None	Varies	Water velocity Suspended solids Pollutant type and level percentage wetting
CHEMICAL			
O_2	Directly proportional	None, if corrosion controlled by O_2 transfer rate	Seawater temperature
CO_2	Little effect	Little effect	NaCl
NaCl	Inversely proportional	Proportional?	Unimportant in open oceans Fresh water inflows Effect of biological Activity
PH	Little effect?	Little effect?	NaCl concentration Pollutant type
Carbonate solubility Pollutants	Little effect Varies	Little effect Varies	Geographical location
PHYSICAL			
Temperature	Directly proportional	Proportional?	Geographical location (not significant for shallow waters)
Water velocity	Little effect	Little effect	Geographical location
Suspended solids	None	Little effect, if any	Geographical location
Percentage wetting	Proportional for tidal and splash zones	Proportional for tidal and splash zones	Location, weather patterns

In practice, there is extensive use of protective coatings (paints), sacrificial coatings (galvanizing), and impressed current cathodic protection to prevent metal corrosion. However, there is evidence that such systems are not always effective, particularly over longer time periods and under less than ideal operational or maintenance conditions (Melchers, 2003a; Melchers, 2003b). In practical terms,

this means that conventional high strength steels corrode at about the same rate as mild structural grade steels, rendering the use of high strength steels in ships problematic (Melchers, 2006a). For advanced socio-economic societies with much infrastructure investment in the coastal region, the corrosion of steels may still be a considerable problem. Due to maintenance costs, cost of protective coatings and increasing concern about the environmental impacts of some corrosion prevention measures, it would be advantageous to use a steel composition such as those metals with a high chromium content that minimizes the rate of corrosion (Melchers, 2008).

1.2.1.2.3 ATMOSPHERIC ENVIRONMENTS

Atmospheric corrosion is a subject of global concern because of its importance to the service life of equipment and the durability of the structural materials. It is a rather complex process involving chemical and electrochemical reactions (Syed, 2006). Since humidity, temperature, contaminants and rainfall vary from place to place, this directly affects the process of atmospheric corrosion of metals, especially in the initial stages (Nishikata *et al*., 1997). The initial products of corrosion form a layer between the metal substrate and the environment, which can greatly affect the subsequent corrosion of the metal. The initial stages of atmospheric corrosion on iron and steels, such as pitting, filiform and nests are localized. The main features of initial corrosion in steels are that filiform corrosion occurs in association with nodules growing on ridge-like filiform corrosion products (Weissenrieder and Leygraf, 2004). In general, atmospheric corrosion of metals might be related with many reactions such as iron oxidation, oxygen reduction and transformation between corrosion products. Factors such as moisture, thickness of electrolyte film, type and concentration of pollutants in the atmosphere plays an important role in the corrosion process. A thin oxide film of Fe_2O_3 could form on the surface of steel in low humidity, which may dissolve in high humidity, so that the substrate might be attacked. Local breakage could take place at spots where the oxide film was locally dissolved or where there are defects (Masuda, 2001; Han *et al.*, 2007).

1.2.2 MICROBIOLOGICALLY INFLUENCED CORROSION

Microorganisms can modify the near-surface environmental chemistry by microbial metabolism and may interfere with electrochemical processes occurring at the metal-environment interface (Chen *et al.*, 2007, El-Shamy *et al*., 2009) leading to either acceleration or inhibition of corrosion (Xu *et al*., 2002; Beech and Sunner, 2004; Rubio *et al*., 2006; Xu *et al*., 2008). This interference often leads to

localized changes in the concentration of electrolyte constituents, pH and oxygen concentration (Xu *et al*., 2002; Xu *et al*., 2008). MIC indicates an increase in corrosion rates due to microbial activity that affects the kinetics of the anodic and/or cathodic reaction, while leaving the corrosion mechanism more or less unchanged (Beech and Sunner, 2004; Mansfeld, 2007).

Microorganisms can affect corrosion behaviour in a number of ways:

- by producing slimes and deposits which give rise to crevice corrosion;
- by creating corrosive conditions through their metabolic products, or by destroying materials added to the system to provide corrosion inhibition; and
- by directly influencing the corrosion reactions (Rothwell, 1979; Adeosun *et al*., 2012; Zhao *et al*., 2012).

The study of MIC of metallic materials has received considerable attention (Dubey and Upadhyay, 2001). MIC can accelerate most forms of corrosion including uniform corrosion, pitting corrosion, crevice corrosion, galvanic corrosion, intergranular corrosion, dealloying, and stress corrosion cracking (Lutterbach and de Franca, 1997). MIC can be a serious industrial problem and affects diverse processes ranging from water distribution in cast iron mains and sewers, to transport of natural gas in steel pipelines (Dubiel *et al*., 2002). Biocorrosion is a result of synergistic interactions between the metal surface, abiotic corrosion products and bacterial cells and their metabolites (Beech and Sunner, 2004). MIC occurs as a consequence of heterogenous biofilm formation which leads to the formation of oxygen concentration gradients, differential aeration cells on the metal surface, selective leaching, cathodic depolarization and corrosion under deposits (Tanji *et al*., 1999; Chen *et al*., 2007).

During the proliferation of microorganisms in an appropriate environment, various types of metabolites are produced which directly participate in electrochemical reactions at the metal/solution interface. Microorganisms produce different oxidizing agents which lead to corrosion (Dubey and Upadhyay, 2001). The presence of electrostatic and adhesion conditions followed by EPS production by microorganisms is unequivocally accepted as a key mechanism facilitating irreversible cell attachment to inanimate surfaces in aqueous environments, thus promoting biofilm development (Characklis and Wilderer, 1989; Beech, 2004; Beech *et al*., 2005; Chongdar *et al*., 2005; Perez *et al*., 2007). The exopolymers usually contain ionic groups, metabolic

end-products like acids, provoking an increase in water content of porous materials and thereby creating concentration cells that facilitate localized corrosion (Cristobal *et al.,* 2006). These biodegradation effects are considerably aggravated when the growth of the aerobic heterotrophic flora is accompanied by that of anaerobic SRB (Tanji *et al*., 1999). Mature biofilms formed on steel are composed of anaerobic microorganisms that are in closest proximity to the steel/biofilm interface, while facultative aerobes are in middle regions of the exopolymeric matrix. Aerobic genera are in the surroundings of the biofilm/solution interface (Perez *et al*., 2007).

The knowledge of bacterial diversity in biofilms is helpful to understand the interactions between corrosive bacteria and metal surface, as well as with other microorganisms, and thus provides the basis for the development of new and better means for the detection and prevention of corrosion (Dubiel *et al*., 2002; Neria-Gonzalez *et al*., 2006). Basic research to increase our understanding of the involvement of microbial species in corrosion and their interaction with metal surfaces and with other microorganisms, will be the basis for the development of new approaches for the detection, monitoring and control of microbial corrosion (Bogan *et al*., 2004). Chemical and microbiological analysis of the milieu, examination of metal characteristics and its corrosive behaviour, examination of corrosion products, etc, are necessary to understand the problem of corrosion (Starosvetsky *et al*., 2007).

1.2.3 MICROBIOLOGICALLY INFLUENCED CORROSION IN METAL ALLOYS

MIC has the potential to affect numerous systems and can be found at any place where an aqeous environment is present. However, it is not exclusive to water-based systems, as it occurs in fuel and lubrication systems as well (Lane, 2005). Although MIC can occur in unexpected places, it tends to occur repeatedly at certain locations. Table 1.3 lists the applications where MIC has been found to be prominent. In general, MIC problem areas for many industries occur more often in the following situations:

- in welds and heat-affected zones;
- under deposits;
- after hydrotesting, if equipment is not drained and dried; and
- when cooling systems are not passivated after turnarounds are complete (Scott, 2004).

Since MIC is a mechanism that often accelerates corrosion, it should be expected to occur more often in metal alloys with susceptibilities to the various forms of corrosion and in environments conducive to biological activity. In general, mild steels can exhibit everything from uniform corrosion to environmentally-assisted cracking, while the remaining alloys usually only show localized forms of corrosion. Titanium alloys have been found to be virtually resistant to MIC under ambient conditions (Lane, 2005).

1.2.3.1 MIC IN MILD STEEL

MIC problems have been widely documented in piping systems, storage tanks, cooling towers, and aquatic structures. Mild steels are widely used in these applications due to their low cost, but are some of the most readily corroded metals (Lane, 2005). Mild steel is a type of steel alloy that contains a high amount of carbon as a major constituent. The ductility, hardness and tensile strength of mild steel are a function of the amount of carbon and other hardening agents present in the alloy (Fadare *et al*., 2011). Poor quality water systems and components with areas that accumulate stagnant water and debris are prone to MIC. In some extreme cases, untreated water left stagnant within mild steel piping has caused uniform corrosion throughout the low lying areas, especially in underground pipes that have been left unused for periods of time. Many power plant piping failures have been found to be the result of introducing untreated water into a system. SRB have been the primary culprit in such cases (Lane, 2005).

Mild steel is known to be attacked by patchy biofilms accumulated on the surfaces due to SRB. In general, biofilm accumulation of SRB forms on an unstable and continuously growing layer of inorganic products on the steel surface (Castaneda and Benetton, 2008). SRB cell adhesion is initiated on amorphous corrosion products followed by coverage of bacterial colonies and corrosion products on the steel surface (Beech and Gaylarde, 1999). The rust layer formed on carbon steels has a complex morphology. It is generally porous, with poor adherence and cracked in its outer part. This renders poor protection to steel against corrosion, because it provides a barrier against the passage of electrolyte and corrosive species towards the metallic substrate (Allam *et al*., 1991; Ma *et al*., 2009). The rust is mainly composed of lepidocrocite (γ-FeOOH), which is usually the first oxyhydroxide formed, and goethite (α-FeOOH). Often, there is also maghemite (γ-Fe_2O_3) (Zou *et*

al., 2011). In marine atmosphere, magnetite (Fe_3O_4) and akaganeite (β-FeOOH) is commonly found (Castano *et al*., 2010).

Table 1.3: Systems with persistent Microbiologically Influenced Corrosion problems (Lane, 2005)

APPLICATION/SYSTEM	PROBLEM COMPONENTS/AREAS	MICROORGANISMS
Pipelines/storage tanks (water, wastewater, gas, oil)	Stagnant areas in the interior Exterior of buried pipelines and tanks, especially in wet clay environments	Aerobic and anaerobic acid producers Sulfate reducing bacteria Iron/manganese oxidizing bacteria Sulfur oxidizing bacteria
Cooling systems	Cooling towers Heat exchangers Storage tanks	Aerobic and anaerobic bacteria Metal oxidizing bacteria Slime forming bacteria Algae Fungi
Docks, piers, and other aquatic structures	Splash zones Just below low tide	Sulphate reducing bacteria
Vehicle fuel tanks	Stagnant areas	Fungi
Power generation plants	Heat exchangers Condensers	Aerobic and anaerobic bacteria Sulphate reducing bacteria Metal oxidizing bacteria
Fire sprinkler systems	Stagnant areas	Anaerobic bacteria Sulphate reducing bacteria

A change to a more corrosion resistant material is not always the most appropriate answer when it comes to solving MIC problems. For example, an upgrade from carbon steel to stainless steel in a nuclear power plant caused a change in MIC problems that in some instances were even more severe (Lane, 2005). Mild steels are normally coated for corrosion protection (Lu *et al*., 1995). Galvanization (zinc coating) is commonly used to protect steel in atmospheric environments. Bituminous coal tar and asphalt dip coatings are often used on the exterior of buried pipelines and tanks, while polymeric coatings are used for atmospheric and water environments. However, biofilms tend to form at flaws in the coating surfaces (Simoes *et al*., 2010). Numerous cases have also been documented where microorganisms cause debonding of coatings from the underlying metal (Little *et al*., 1991). Delamination of the coating, in turn, creates an ideal environment for further microbial growth (Lane, 2005).

The use of organic inhibitors that absorbs on the metal surface is effective in preventing corrosion. The adsorption is influenced by factors such as the nature and surface charges on the metal, the type of aggressive media, the structure of inhibitor and the nature of its interaction with the metal surface (Lowmunkhong *et al*., 2010). Another way to control MIC is exploitation of other microorganisms, which may inhibit corrosion causative microbes. For example, Jayaraman *et al*. (1999a) reported inhibitory effects on axenic aerobic biofilms on corrosion of copper and aluminium. Pederson and Hermansson (1989; 1991) reported an eight fold corrosion inhibition of mild steel by employing anaerobic bacteria under favorable conditions. The formation of biofilms by microbes inhibitory to the MIC process on metal surfaces has been reported by many other workers (Little and Ray, 2002; Ornek *et al*., 2002; Zuo *et al*., 2005).

1.2.3.2 MIC IN STAINLESS STEEL

Stainless steel is an alloy that consists of 10.5% or more chromium and more than 50% iron (Newson, 2002; Lane, 2005). The chromium reacts with oxygen to form a tough, adherent, invisible, passive layer of chromium oxide film on the steel surface. Generally, an increase of chromium content improves the corrosion resistance of steels (El-Meligi, 2010). Stainless steels have suffered MIC problems under the same sets of conditions as mild steels, primarily in situations where water accumulates. There are two notable problems that have surfaced with MIC of stainless steels (Little *et al*., 1991; Lane, 2005). First, stainless steels corrode at an accelerated rate, primarily through pitting or crevice corrosion, which occurs at low lying areas, joints, and at corner locations. This has been found to occur in tanks and piping systems that were hydrotested using well water, and then put in storage before service without using biocides or drying the system to prevent microbial growth. The second MIC problem is that corrosion occurs adjacent to weldments (Lane, 2005).

Although the mechanisms of MIC are largely based on speculation at present, progress has been made in identification of metallurgical features associated with MIC. In the case of stainless steels, weld and heat affected zones are most susceptible to MIC-associated pitting corrosion (Wahid *et al*., 1993; Geesey *et al*., 1996). The majority of chemical evidence attributing certain types of localized corrosion to microorganisms on metal surfaces has been based on data obtained from surface deposits which have accumulated long after the corrosion reaction was initiated. Unfortunately, the chemistry of the deposits does not necessarily offer insight to the critical, initial microbially-

mediated reactions that compromise the protective surface oxide film in the presence of a developing biofilm (Geesey *et al*., 1996).

1.2.4 THE ROLE OF MICROORGANISMS IN METAL CORROSION

MIC occurs as microbes grow and metabolize in either aerobic or anaerobic conditions (Augustin and Ali-Vehmas, 2004). The microorganisms metabolic reactions attributable to metallic corrosion involve sulphide production, acid production, ammonia production, metal deposition, and metal oxidation and reduction (Lane, 2005). Table 1.4 lists some specific microorganisms that have been attributed to MIC along with their characteristics. Metabolism of microbes results in the production of gelatinous slimes and metabolites (e.g. organic acids, sulphates and sulphides) that lead to aggressive environments for metals. Bacteria are amongst the most successful living organisms and their ubiquity ensures that humans are obliged to live in constant and intimate contact with a wide variety of species (Augustin and Ali-Vehmas, 2004). Bacteria are considered the primary colonizers of inanimate surfaces in both natural and man-made environments (Beech and Sunner, 2004; Lane, 2005). Energy is generated for growth by the oxidation of ferrous ions to ferric ions and may participate in rusty slime formation. Therefore, the metal surface may be modified because of bacterial growth and activity which influences the corrosion process (Ismail *et al.*, 1999; Chen *et al.*, 2007). The majority of MIC investigations have addressed the impact of pure or mixed culture bacterial biofilms on corrosion behaviour of iron, copper, aluminium and their alloys (Beech and Sunner, 2004).

Bacteria identified thus far on corroding surfaces encompass a range of species with different metabolic properties, including the abilities to reduce oxygen, sulphate, sulphite, and ferric iron; oxidize sulphide and ferrous iron; ferment; and/or produce acid. SRB, including *Desulfovibrio desulfuricans*, have long been associated with the corrosion of steel and have been the focus of most MIC research (Lee *et al*., 2006). Bacteria belonging to the genus *Desulfovibrio* (Table 1.4) perform the role of a cathodic depolarizer by removing the hydrogen from the metallic surface with the help of the enzyme, hydrogenase and thus promote the process of corrosion. Besides these anaerobes, a number of soil inhabiting microbes are suspected of playing an important role in the process of corrosion of buried structures (Dayal *et al.*, 1988).

Table 1.4: Bacteria known to cause MIC (Modified from Jones ,1995)

GENUS OF SPECIES	***pH***	***TEMPERATURE (°C)***	***OXYGEN REQUIREMENT***	***METALS AFFECTED***
Desulfovibrio sp.	4-8	10-40	Anaerobic	Iron, steel, stainless steels, aluminum, zinc, and copper alloys
Desulfotomaculum sp.	6-8	10 – 40 (some 45 – 75)	Anaerobic	Iron and steel; stainless steels
Desulfomonas sp.		10 – 40	Anaerobic	Iron and steel
Thiobacillus Thioxidans	0.5–8	10 – 40	Aerobic	Iron and steel, copper alloys
Thiobacillus ferrooxidans	1-7	10-40	Aerobic	Iron and steel
Gallionella sp.	7-10	20-40	Aerobic	Iron and steel
Sphaerotilus sp.	7-10	20-40	Aerobic	Iron and steel

1.2.4.1 SULPHATE-REDUCING BACTERIA (SRB)

SRB such as *Desulfovibrio* sp., *Desulfotomaculum* sp. and *Desulfomonas* sp. in Table 1.4 are mostly responsible for the corrosion of cast iron, carbon steel, low allow steels, stainless steels, high nickel alloys and copper alloys in both aquatic and terrestrial environments, under anoxic and oxygenated conditions (Zinkevich *et al.*, 1996; Beech and Gaylarde, 1999; Zuo *et al*., 2004; Lopes *et al*., 2006; El-Shamy *et al.*, 2009; Wen and Hong-Bo, 2011). It has been proposed that the main product of sulphate respiration, sulphide, contributes to the further corrosion of steel (Lee *et al*., 2006). The presence and metabolic activity of anaerobically growing microbes leads to the production of hydrogen sulphide and changes in pH levels (Fonseca *et al.*, 1997; Sand, 1997; Wen and Hong-Bo, 2011). Biogenic sulphide production leads to health and safety problems, environmental hazards and severe economic losses due to reservoir souring (increased sulphur content) and the corrosion of equipment (Beech and Gaylarde, 1999).

SRB are a group of phylogenetically diverse anaerobic microorganisms which have been regarded as physiologically and ecologically similar. The presence of SRB in natural and man-made systems is of great concern to industries worldwide. The oil, gas and shipping industries are seriously affected due to the involvement of these bacteria in hydrocarbon reservoir souring, corrosion and by the sulphides generated by SRB (Beech and Gaylarde, 1999; Fang *et al*., 2002; Lopes *et al*., 2006; Ilhan-Sungar *et al*., 2007; Gonzalez-Rodriguez *et al*., 2008). It has been reported that different

genera and species of SRB vary in their ability to influence metal deterioration (Feio *et al.,* 1998). SRB reduce oxidized sulphur compounds, such as sulphate, sulphite, thiosulphate, thionic acids, and sulphur, to H_2S. The reaction is used as a sink for electrons originating from organic substrates. It enables SRB to grow oxidatively under anaerobic conditions. Sulphate, as the most important sulphur compound, is ubiquitously present in water. Thus, H_2S may be produced whenever anaerobic conditions occur. However, recent work has shown that SRB may be oxygen-tolerant or even may be able to use some oxygen (under reduced partial pressure) for oxidative metabolism. In addition, H_2S is a result of amino acid degradation (Sand, 1997). Additional studies have shown that other types of bacteria, like oxygen-consuming and iron-reducing bacteria, can inhibit corrosion. Bacterial Fe (III) respiration protects steel from corrosion, in part because the resulting high concentration of soluble Fe (II) scavenges oxygen in the water column. Some bacteria can prevent steel from corroding in certain conditions, therefore there is potential for developing corrosion-control methods by manipulating bacterial populations to favour certain organisms over others (Lee *et al*., 2006).

These microorganisms can coexist in naturally occurring biofilms with a wide bacterial community including fermentative bacteria, often forming synergistic communities that are capable of affecting electrochemical processes through co-operative metabolism (Pankhania *et a*l., 1986; Gonzalez-Rodriguez *et al.,* 2008). Their diverse metabolic capabilities allow them to carry out various interactions in different ecological niches such as marine and estuarine sediments and biofilms (Pankhania *et al*., 1986). SRB needs a supply of sulphates for reduction of sulphides, organic carbon and nutrients for their metabolism. Some species have a high requirement for inorganic iron. The absence of any of these will reduce considerably or curtail their activity. Inorganic nutrients such as nitrogen and phosphorous typically are available in waters as nitrates, nitrites, ammonium, phosphates and orthophosphates (Melchers, 2007). SRB are capable of growth on a wider range of substrates than had been previously appreciated and are thus very flexible ecologically (Pankhania *et al*., 1986).

Although SRB are often considered to be strictly anaerobic, anaerobic conditions may also occur on the corrosion surface due to the presence of other aerobic bacteria producing anoxic pockets. Once colonization has begun, bacterial growth is further promoted due to the spread of reducing conditions (Tanji *et al.,* 1999; Seth and Edyvean, 2006; Sheng *et al*., 2007). Some genera tolerate oxygen and at low dissolved oxygen concentrations whereby certain SRB are able to respire with

Fe^{3+} or even oxygen with hydrogen acting as an electron donor (Beech and Gaylarde, 1999). SRBs are distinguished by their ability to conduct dissimilatory sulfate reduction to sulfide which may be released as H_2S or iron sulfide (Fe_2S) in the case of ferrous metals (Figure 1.2) (Obuekwe *et al*., 1981a; Jain, 1995). SRB exert a significant ecological, economic and environmental impact (Jain, 1995). An understanding of the ecology of SRB in subterranean environments is essential for evaluation of the adverse effects associated with their hydrogen sulphide production (Motamedi and Pedersen, 1998).

In marine ecosystems, SRB contribute significantly (50%) to the mineralization of organic matter (Haouari *et al.*, 2006; Francisco *et al*., 2012). Hydrogenotrophic SRB of the family Desulfovibrionaceae within the Deltaproteobacteria have been found as common inhabitants of these ecosystems (Haouari *et al.*, 2006). Marine SRB, among which the *Desulfovibrio* and *Desulfotomaculum* genera are dominant, make up an ecologically and morphologically heterogeneous group of microorganisms. The main property of those either obligate or facultative anaerobic bacteria populations is their active use of sulphate as a final electron acceptor during anaerobic respiration. The final product of this respiration is H_2S, which is discharged into the environment (Javaherdashti, 1999; Mudryk *et al*., 2000). H_2S is a very strong reducing agent and highly toxic to both bacteria and higher forms of life (Jain, 1995).

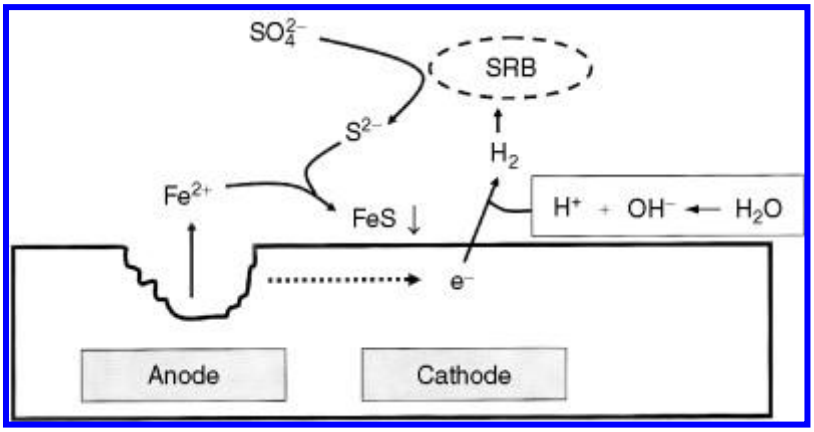

Figure 1.2: Influence of SRB on corrosion of ferrous metals (Barton and Fauque, 2009)

Where concentrations of H_2S are very high, this can penetrate SRB cell membranes and thus impede their metabolic activity (Mudryk *et al*., 2000). The SRB activity can (i) induce microbial corrosion by cathodic depolarization, (ii) produce H_2S gas which is toxic to plants, animals and

humans, and (iii) result in souring of oil and gas, and rock-pore blockage by bacterial cells and precipitates of FeS (Jain, 1995). SRB utilize a very wide spectrum of different low molecular compounds as electron donors and also as carbon and energy sources (Mudryk *et al*., 2000). The rate of metabolism of SRB and other bacteria depends, in part, on the availability of necessary nutrients. For seawater, nitrogen, inorganic carbon and iron may be limiting. In the corrosion process, the latter two are available through other bacteria in the consortia and from the corrosion process itself. Nitrogen as ammonium, nitrites and nitrates are present only in very low concentrations in natural seawaters (Melchers and Jeffrey, 2008). The presence of nitrate may pose a specific stress to SRB as nitrate has been observed to suppress sulphate reduction activity *in situ*. However, it has been suggested that nitrite, an intermediate that transiently accumulates during nitrate reduction, is directly responsible for the inhibition of sulphate reduction activity (He *et al*., 2006).

SRB synthesise numerous enzymes that catalyse sulphate reduction. The following enzymes play a major part in sulphate activation and reduction: pyrophosphatase, ATP sulphurylase, bisulphate reductase, desulphoviridin, desulphorubidin and desulphofuscidin (Gibson, 1990; Sass *et al*., 1992; Visscher *et al*., 1992). Sulphate and organic matter concentration, sedimentation rate, turbulence, bioturbulence temperature, salinity and hydrostatic pressure are the main environmental factors controlling the numbers and distribution of SRB and the rate of bacterial sulphate reduction (Westrich and Berner, 1998; Mudryk *et al*., 2000). SRB, which generate large amounts of toxic H_2S in aquatic ecosystems, are important not only for ecological reasons but also vital in the economy (Mudryk *et al*., 2000). Control of biogenic sulphide production decreases operating costs and can be achieved through the application of biocides, nitrate or nitrite (Greene *et al.*, 2006). Nitrate injection changes the microbial community in the subsurface from mainly SRB to one enriched in nitrate-reducing bacteria (NRB), which include the nitrate-reducing, sulphide-oxidizing bacteria (NR-SOB) that oxidize H_2S directly and the heterotrophic NRB, which compete with SRB for degradable organic electron donors and thus potentially prevent SRB metabolism (Hubert and Voordouw, 2007).

Environmental regulations and development of oil reservoirs in environmentally sensitive areas have spurred the development of easily degradable “green” biocides that are less toxic to higher, non-target organisms, like fish. Nitrite, a specific metabolic inhibitor of SRB is also relatively non-toxic and inexpensive and has been successfully used to inhibit sulphide production in oil field

settings (Greene *et al.,* 2006). The biochemistry and physiology of SRB should be considered when determining their aggressiveness towards metals. It has also been shown that different genera and even species of sulphate-reducers can vary in their ability to deteriorate carbon steel. SRB are often present in biofilms formed on steel surfaces thriving at the substratum/biofilm interface (Zinkevich *et al.,* 1996).

1.2.4.2 METAL-REDUCING BACTERIA (MRB)

Microorganisms are known to promote corrosion of iron and its alloys through reactions leading to the dissolution of corrosion-resistant oxide films on the metal surface. This results in the protective passive layers on stainless steel surfaces for example, being lost or replaced by less stable reduced metal films that allow further corrosion to occur (Beech and Gaylarde, 1999). Iron/manganese oxidizing bacteria have been found in conjunction with MIC, and are typically located in corrosion pits on steels. Some species are known to accumulate iron or manganese compounds resulting from the oxidation process. High concentrations of manganese in biofilms have been attributed to the corrosion of ferrous alloys, including pitting of stainless steels in treated water systems. Iron tubercles have also been observed to form as a result of the oxidation process (Lane, 2005).

Despite its widespread occurrence in nature and likely importance to industrial corrosion, bacterial metal reduction has not been seriously considered in corrosion reactions until recently. Numerous types of bacteria, including those from the genera *Pseudomonas* and *Shewanella* are able to carry out manganese and/or iron oxide reduction and have been shown to influence corrosion reactions (Obuekwe *et al*., 1981b; Myers and Nealson, 1988; Beech and Gaylarde, 1999). *Hafnia alvei*, *Desulfovibrio desulfuricans*, *Bacillus* sp. and *Pseudomonas* sp. are reported to influence the corrosion behaviour of mild steels and stainless steels in marine habitats. Corrosion of iron and nickel has been reported to have increased in the presence of *Pseudomonas* sp. and *Serratia marcescans*. On the other hand, *Pseudomonas fragi*, *Escherichia coli* DH5α and *Pseudomonas flava*, when present as living biofilms, are known to inhibit corrosion (Chongdar *et al*., 2005). It has been shown that in cultures of *Shewanella putrefaciens*, iron oxide-surface contact was required for bacterial cells to mediate reduction of these metals. The rate of reaction depended on the type of oxide film under attack (Beech and Gaylarde, 1999).

1.2.4.3 METAL-DEPOSITING BACTERIA (MDB)

Bacteria of the genera *Siderocapsa*, *Gallionella*, *Leptothrix*, *Sphaerotilus*, *Crenothrix* and *Clonothrix* participate in the biotransformation of oxides of metals such as iron and manganese (Guonot, 1994; Beech and Gaylarde, 1999; Wen and Hong-Bo, 2011; Lin and Ballim, 2012). *Gallionella* (Table 1.4) and *Leptothrix* are neutrophilic iron-oxidizing bacteria that occur in various forms and can occupy many different environmental niches. These bacteria thrive in conditions with a pH range of 6.0-8.0, although they can survive in a more acidic or basic environment. They can also be autotrophic or mixotrophic. *Gallionella ferruginea* is a gradient organism that develops at the anoxic-oxic interface where conditions are neither strongly reducing nor highly oxidizing. These cells are usually kidney or bean-shaped but they may also be rod shaped. The genus *Leptothrix* is characterized by trichomes of cylindrical or rod-shaped, colorless cells within a sheath encrusted with iron or manganese oxide. These bacteria are unicellular but usually occur in chains and are mostly found in fresh water. *Leptothrix* is often confused with the genus *Sphaerotilus* as they are very similar in morphology. *Sphaerotilus* cells (Table 1.4) can be either free-floating or attached in chains or trichomes. They are colorless rods or ellipsoids, are surrounded by a firm sheath, and are found in stagnant or running water (Schiermeyer *et al*., 2000).

Iron is the fourth most abundant element in the Earth's crust and therefore, the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) by bacteria has proven to be very important in the area of biocorrosion (Tatnall, 1981; Keevil *et al*., 1989; Lutey, 1992; Schiermeyer *et al*., 2000). Since iron-oxidizing bacteria are abundant world-wide, microbial corrosion has become a significant and widespread problem in water, aqueous waste systems, and the petrochemical industry. The iron-oxidizing bacteria indicate their presence with large reddish-brown deposits or rust-coloured streaks most often occurring on stainless steel. These iron-oxidizers can concentrate chlorides into ferric and manganic chloride-rich deposits. This acts like dilute HCl and causes general corrosion of steel. They usually form thick, bulky deposits which can lead to highly localized damage, such as subsurface pit cavities and stress corrosion cracking (Schiermeyer *et al*., 2000). Also, with the ferric hydroxide they produce, they can form extensive deposits called tubercles containing microorganisms, inorganic and organic materials (Dickinson *et al*., 1997; Beech and Gaylarde, 1999; Schiermeyer *et al*., 2000).

Microscopy has detected the presence of sheathed filamentous bacteria in naturally formed corrosion deposits and thus proposed that it may play a role in the corrosion of steels (Tatnall, 1981; Keevil *et al*., 1989; Lutey, 1992; Beech and Gaylarde, 1999). The only metals resistant to damage by iron-oxidizing bacteria *Gallionella* are titanium and other noble metals (Schiermeyer *et al*., 2000). The resistance to corrosion of alloys such as stainless steels is due to the formation of a thin passive oxide film. The formation of organic and inorganic deposits by MDB on the oxide surface compromises the stability of this film (Dickinson *et al*., 1997; Beech and Gaylarde, 1999; Schiermeyer *et al*., 2000). Dense accumulations of MDB on the metal surface may thus promote corrosion reactions by the deposition of cathodically-reactive ferric and manganic oxides and the local consumption of oxygen by bacterial respiration in the deposit (Dickinson *et al*., 1997; Beech and Gaylarde, 1999). Some bacteria are known to adhere preferentially to corrosion products and thus will be present in high numbers even when playing no role in the primary corrosion process (Beech and Gaylarde, 1999).

1.2.4.4 SLIME-PRODUCING BACTERIA

Aerobic microorganisms that produce copious quantities of extracellular polysaccharides (EPS) during growth in biofilms have been implicated in localized attack of stainless steels (Pope *et al*., 1984; Beech and Gaylarde, 1999). The slime controls permeation of nutrients to the cells and may break down various substances, including biocides. Slime formers have been responsible for the decreased performance of heat exchangers as well as clogging of fuel lines and filters. They can prevent oxygen from reaching the underlying metal surface, creating an environment suitable for anaerobic organisms (Lane, 2005).

Slime-forming microorganisms that have been recovered from sites of corrosion on stainless steels include *Clostridium* sp., *Flavobacterium* sp., *Bacillus* sp., *Desulfovibrio* sp., *Desulfotomaculum* sp. and *Pseudomonas* sp. As little as 10 ng cm^{-2} EPS has been reported to provoke the onset of MIC on stainless steel in natural seawater; cathodic protection of the stainless steels, used to prevent corrosion, actually increased the amount of EPS in the biofilm (Keevil *et al*., 1989). However, the role of EPS in MIC of stainless steels remains obscure. It has been postulated that they are not sufficient to induce biocorrosion of stainless steels unless aided by the presence of a biocatalyst of oxygen reduction, which could be oxido-reductase enzymes entrapped in the biofilm. EPS has even been suggested to protect metal surfaces from corrosion. A bacterial consortium consisting of a

thermophilic *Bacillus* sp. and *Deleya marina* produced a metal-binding EPS that reduced the rate of corrosion of carbon steel by 94%. Such a mechanism may be responsible for the protection microorganisms afford to mild steels under certain conditions (Beech and Gaylarde, 1999).

Most bacteria can attach to surfaces efficiently when they are viable and in a metabolically active state. Wild *Bacillus* strains form elaborate multicellular communities that display conspicuous architectural features such as fruiting-body-like aerial projections that extend from the surfaces of the biofilms. In addition, some *Bacillus* species are able to produce highly hydrophobic spores able to adhere firmly to various inert substrata. Once this first step of adhesion has been completed, colonization may occur when environmental conditions become favorable to spore germination (Korenblum *et al*., 2005). Microbiological evaluation of the corrosion deposits showed that high numbers of bacteria were not always related to pitting and that the range of cultured bacterial species was quite variable (Beech and Gaylarde, 1999).

1.2.4.5 ACID-PRODUCING BACTERIA (APB)

Acid producing bacteria are a group of heterotrophs that are able to produce organic acids as metabolic products when grown under reduced environmental conditions (Lin and Ballim, 2012). Bacteria can produce copious quantities of either inorganic or organic acids as by-products of metabolism, leading to serious corrosion damage to equipment (Soracco *et al*., 1988; Lin and Ballim, 2012). Acidophilic sulphur oxidizing bacteria (SOB), such as *Thiobacillus* sp. (Table 1.4) are chemoautotrophic bacteria, which obtain their energy for growth and other maintenance from oxidative conversion of sulphur and/or its other reduced compounds. These compounds are chemically or microbiologically oxidized into sulphate, ultimately producing highly corrosive sulphuric acid (Beech and Gaylarde, 1999; Dubey and Upadhyay, 2001).

These microbes can cause severe corrosion damage to mining equipment. Organic acid-producing bacteria were suggested as the primary cause in a case of carbon steel corrosion in an electric power station. They were the only group of culturable microorganisms whose abundance was correlated positively with corrosion (Beech and Gaylarde, 1999). These bacteria are more apt to be found in closed systems including gas transmission lines and sometimes closed water systems (Lane, 2005).

1.2.4.6 FUNGI

Fungi are well-known to produce organic acids and are therefore capable of contributing to the biocorrosion of steel and aluminium (Shreir, 1963). Much of the published work on biocorrosion of aluminium and its alloys have implicated fungal contaminants of jet fuel such as *Hormoconis resinae*, *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. The large quantities of organic acid by-products excreted by this fungus selectively dissolve or chelate the copper, zinc and iron at the grain boundaries of aircraft aluminum alloys, forming pits which persist under the anaerobic conditions established under the fungal mat. Growth of this and other fungi in diesel fuel storage tanks can produce large quantities of biomass and this may provoke crevice attack on the metal (Lane, 2005).

1.2.4.7 MICROBIAL CONSORTIA

In any natural environment, MIC is probably not the result of one single organism acting by a single mechanism; rather, it is a result of a consortium of different microorganisms acting *via* different mechanisms (Angell and White, 1995). The main types of bacteria associated with metals in terrestrial and aquatic habitats are sulphate-, iron- and CO_2-reducing bacteria, sulphur-, iron- and manganese-oxidizing bacteria (Beech and Sunner, 2004). The major groups of microorganisms associated with anaerobic corrosion are the SRB (Dinh *et al*., 2004). The latter microorganisms can coexist in naturally occurring biofilms with a wide bacterial community including fermentative bacteria, often forming synergistic communities that are able to affect electrochemical processes through co-operative metabolism (Miranda *et al*., 2006).

Microorganisms are almost never found in nature as pure species and, while laboratory studies on isolated pure cultures are essential to the understanding of MIC, the role of microbial consortia is becoming increasingly recognized (Dunne, 2002). The acids produced by APB serve as nutrients for SRB and methanogens and it has been suggested that SRB proliferate at sites of corrosion due to the activities of APB. Consortia of MDB and SRB often exist as biofilms on corroding metal surfaces (Videla and Characklis, 1992). It has been proposed that oxygen consumption by MDB creates redox conditions favorable for the growth of SRB and the joint action of MDB and SRB may promote the breakdown of the passive film on stainless steels (Beech and Gaylarde, 1999).

1.2.5 BIOFILMS IN METAL BIOCORROSION

Any surface that comes into contact with water can be colonized with microorganisms, resulting in a sequential event leading to the formation of biofilms (Beech and Cheung, 1995). Biofilms comprise of microorganisms that produce EPS, forming highly hydrated structures on the substratum (Lin and Ballim, 2012). Therefore, biofilms are defined as functional consortia of microorganisms organized within extensive polymer matrices (Jayaraman *et al.*, 1998). It has been estimated that 99% of all bacteria in natural environments exist within a biofilm, or at least reside at the surface (Macdonald and Brozel, 2000; de Carvalho, 2007). Microorganisms colonize a surface and form a mono- or multilayer of cells. These organisms ultimately occupy the entire surface, but patchy growth is also often observed (Characklis and Wilderer, 1989). Biofilms are a kind of microbial consortia which play an important role in the biogeochemical processes of the biosphere (Nikolaev and Plakunov, 2007).

Biofilm-forming communities are subject to variations and are dependant on environmental conditions (water quality, temperature, etc.), as well as type of substratum present for colonization (Lutterbach and de Franca, 1997; Macdonald and Brozel, 2000; de Carvalho, 2007). The aqueous environment and the substratum influences the selection of microorganisms that will predominate in the developing biofilm (Macdonald and Brozel, 2000; de Carvalho, 2007). Biofilms can develop on metal surfaces in natural environments and have been thought to accelerate the rate of corrosion (Jayaraman *et al.*, 1997b; Lutterbach and de Franca, 1997; Lin and Ballim, 2012). Corrosion occurs as a consequence of non-uniform biofilm formation and microcolony formation of oxygen concentration gradients and differential aeration cells near the metal surface (Figure 1.3) (Jayaraman *et al.*, 1997c). Problems associated with biocorrosion due to biofilm formation are widespread and have serious economic and safety implications. However, it still remains to be elucidated how much a role MIC plays in the corrosion process and what are the key mechanisms involved (Beech, 1996).

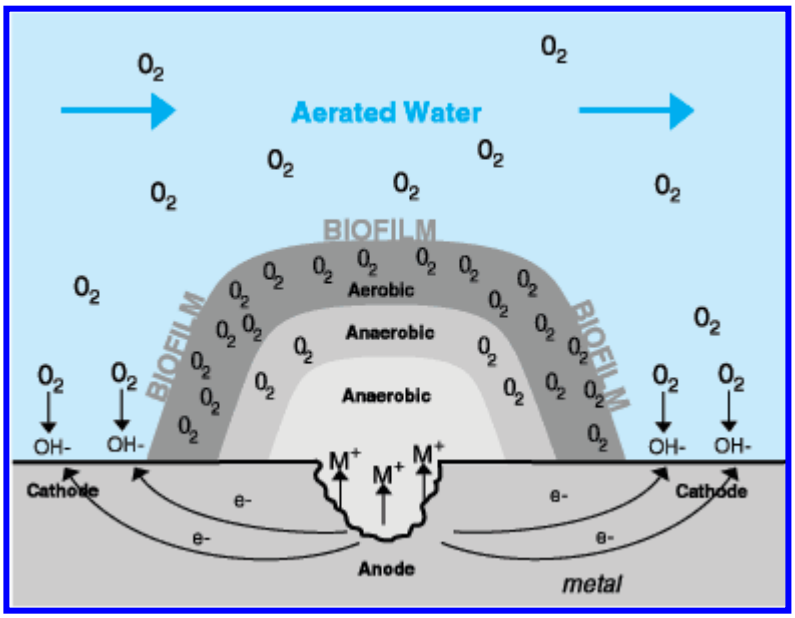

Figure 1.3: Non-uniform (patchy) colonization by bacteria results in differential aeration cells. This schematic shows pit initiation due to oxygen depletion under a biofilm. (Borenstein, 1994)

Development of biofilms occurs in different shapes, depending on the chemical characteristics of the water and the dynamics of the system. The structure of the biofilm, in turn, has a direct influence upon the mechanisms of corrosion (Lutterbach and de Franca, 1997). Biofilms create microenvironments for a consortium of bacteria residing on a substrate. These microenvironments include variations in pH, nutrient concentrations, and oxygen levels as seen in Figure 1.3 (Else *et al.,* 2003; Lopes *et al*., 2006). The biofilm and its microenvironment are continuously exchanging specific components in this relatively complex way (Characklis and Wilderer, 1989). The structure of biofilms allows the flow of nutrients, enzymes, metabolites, waste products and other solutes. Under MIC conditions, the colonization and growth of bacteria on the metal surface give rise to a biofilm which displays a non-uniform coverage on the surface as seen in Figure 1.3 (Else *et al.*, 2003; Landoulsi *et al.,* 2008). MIC also includes production of microbial metabolites at one location which diffuse to a corrosion site, possibly at another location. MIC of metal surfaces results in pitting, crevice corrosion, under-deposit corrosion and selective leaching (Else *et al.*, 2003).

Biofilms exhibit complex three-dimensional structures, such as channels for fluid influx, and differential gene expression in different regions (Creepi, 2001). Although bacterial EPS have been shown to exhibit selectivity in complexing metal ions, in many cases the type of macromolecules

playing a key role in metal binding has not been determined (Beech and Cheung, 1995). The initial bacterial attachment to the metal surface and the subsequent formation of biofilm are dependant on the surface characteristics of the substratum, including metal surface free energy, roughness and hydrophobicity, as well as metallurgical features. Bacteria tend to preferentially colonize onto the grain boundaries of steel. This could cause localized corrosion, and the resulting corrosion may further promote the patchy adsorption of microbes. On the other hand, biochemical characteristics of the microbial surface and the EPS are equally crucial to the biofilm formation (Fang *et al.*, 2002). EPS are primarily composed of polysaccharides, uronic acid sugars and proteins, containing functional groups such as carboxylic acid and amino acid groups, which could be acidic and capable of binding metal ions (Lin and Ballim, 2012). Thus, EPS can also affect the electrochemical characteristics of the metal surface and play an important role in their corrosion (Fang *et al.*, 2002).

Biofilm formation is the key to the alterations of conditions at a metal surface before the initiation of biocorrosion processes (Guiamet *et al.,* 1999). Since bacteria can coat metals with a regenerative biofilm, it is becoming evident that this may be an alternative means for corrosion prevention. *Pseudomonads* and other aerobic bacteria have been shown to both increase and decrease the rate of metal corrosion through polymer production during biofilm formation. Aerobic bacteria can create localized anaerobic niches in a biofilm, which allows SRB to colonize and increase corrosion. Under certain favourable conditions, *Pseudomonads* have also been able to prevent corrosion. It has been suggested that the presence of a biofilm may act as a diffusional barrier to the corrosive substances and prevent them from reaching the metal surface (Jayaraman *et al.,* 1997b). Thus, preventing the initial step of biofilm formation (bacterial adhesion) on the metal surfaces would be one of the potential ways to avoid biocorrosion. Otherwise, the adhesion of microorganisms to surfaces affects a wide variety of industrial systems and operations, such as cooling water systems, water injection for oil recovery, reverse osmosis membranes, the permeability of oil reservoirs, etc. The biofilm containing 95% of water in a matrix of EPS in which bacterial cells and inorganic detritus are suspended, is the key factor to explain the physical, chemical and biological alterations at the metal/solution interface prior to the induction of localized attack or to corrosion inhibition (Guiamet *et al.,* 1999).

Biofilm accumulation may cause biological fouling, heat transfer losses, product quality deterioration and biocorrosion (Johansson *et al.,* 1999). On metal surfaces, microbial growth may alter the microclimate locally, e.g., by producing oxygen concentration gradients in pH, excreting

organic acids or allowing the proliferation of certain anerobic bacteria within the biofilm (Lin and Ballim, 2012). Investigations of the interactions between bacteria and steel during the early stages of biocorrosion could yield more insight into the reaction mechanisms of the process. Recent investigations indicate that not only the identified corrosion-causing bacteria, but also many others may be involved in biocorrosion. However, monitoring the first subtle changes is challenging due to the inert substrates and the complexity of the biological growth processes (Johansson *et al.,* 1999).

1.2.6 METHODS TO EXAMINE MICROBIOLOGICALLY INFLUENCED CORROSION

1.2.6.1 MICROSCOPY

Traditional culture techniques have yielded valuable information about microbial interactions within the environment. However, only a small fraction of microorganisms can be cultured from environmental samples (Wen and Hong-Bo, 2011). The most common method for enumeration and morphological observation of microorganisms on metal surfaces is microscopy. This includes scanning electron microscopy (SEM) and fluorescence microscopy. SEM provides information about the structure of the biofilm and can reveal the presence of different kinds of EPS (Silva *et al*., 2007). Traditional SEM allows for analyses of biofilm development, composition, distribution and relationships to substratum or corrosion products (Little *et al*., 2006). Elements in corrosion deposits can provide information as to the cause of corrosion. Energy dispersive X-ray analysis coupled with SEM can be used to determine the elemental composition of corrosion deposits. Elements in corrosion deposits can provide information as to the cause of corrosion (Little *et al*., 2006).

Applied microscopic techniques provide a qualitative assessment, while surface chemical techniques provide qualitative and quantitative estimations of the characteristics of the biofilm formation, transport and electrochemical corrosion processes (Malucknov, 2012). Atomic force microscopy (AFM), environmental scanning electron microscopy (ESEM) and confocal laser scanning microscopy (CLSM) can be used for biofilm observation in real time without changing the sample structure (Malucknov, 2012; Sheng *et al*., 2012). The ESEM technique ensures detailed observation of biofilms with high resolution images. CLSM and AFM provide clean, three dimensional images of living biofilms in real time (Malucknov, 2012). CLSM investigates the distribution and viability of bacteria adsorbed on the metal surface by staining with a combination

dye such as LIVE/DEAD Baclight bacteria viability kits. The viable cells appear green, while non-viable or membrane compromised cells appear red. AFM is used to obtain biofilm images with pits displayed on the surface of the metal. It is also used to image biofilm topography, to quantify the surface roughness and allows for quantification of pit depth (Sheng *et al*., 2012). Microscopically obtained data on the type of microorganisms, combined with chemical analyses of metal surfaces and electrochemical measurements, provide information about the chemical composition of the corrosion products and microbiological deposits. These data can be used to estimate the level of corrosion (Malucknov, 2012).

1.2.6.2 ELECTROCHEMICAL TECHNIQUES

A wide range of electrochemical techniques, such as corrosion and redox potential measurements, Tafel and potentiodynamic polarization, linear polarization and electrical resistance probes, as well as several modern electrochemical techniques, including alternating current methods or electrochemical noise have been critically reviewed in previous studies in relation to their use in biocorrosion evaluation (Videla and Herrera, 2005). Electrochemical Impedance Spectroscopy (EIS) is used to investigate the electrochemical properties and corrosion behavior of the metal surface (Sheng *et al*., 2012). The electrochemical reactions involved in the corrosion processes caused by microorganisms can be clarified by obtaining information on the chemical structure of corrosive products and microbiological layers, as well as quantifying the corrosion rate (Malucknov, 2012).

1.2.6.3 MOLECULAR TECHNIQUES

Culture-independent approaches to microbial community analyses and molecular description now dominates all microbial ecology literature (Martiny *et al.*, 2005). Since 1985, DNA sequencing technology has been used to study microbial ecology and evolution, whereas the research on microbial diversity has entered a stage that focuses on the development of methods and techniques of the molecular ecological technology of microorganisms (Wen and Hong-Bo, 2011).

Molecular techniques involving bacterial DNA and RNA are among the most exciting and promising techniques in MIC research. They offer the potential to: (a) identify dominant bacteria in a given ecological system without the serious limitations of standard viable counting techniques; (b)

calculate the proportion of MIC-contributing bacteria in the total population; (c) identify bacteria that are susceptible or resistant to antimicrobials; (d) assess the changes in the overall population caused either by the use of biocides or nutrient modifications; and (e) achieve a more reliable sampling, not affected by time or transport factors (Videla and Herrera, 2005).

Classical microbiological identification methods are based on the cultivation of the organism. Since all media are selective to some degree and not all bacteria or other microorganisms are culturable, viable counts rarely represent all microorganisms present in the sample (Dunne, 2002). Polymerase chain reaction (PCR) allows for identification of biofilm organisms without prior cultivation. The method allows for establishment of genetic fingerprints of bacteria, protozoa, fungi and algae that may be present in the sample (Little *et al*., 2006). 16S rRNA sequencing and analysis method is an important way to infer phylogeny of bacteria and their evolution, which gives comparative analyses of the homology of 16S rRNA sequences of different bacteria. The evolutionary distance calculated will determine the types of microbes that may exist in environmental samples (Wen and Hong-Bo, 2011).

The genetic material (DNA, RNA) inside the bacteria is unique for all individual species. By using the sequences of base pairs in the 16S rRNA, probes can be designed to target individual groups, generas or strains of bacteria (Clarridge, 2004). With the use of rRNA directed and fluorescence labeled oligonucleotide probes, it is possible to detect and localize microorganisms that may not be cultivable and place them in a phylogenetic tree (Little *et al*., 2006). Although the preparation steps are different for the individual oligonucleotide probes, the cells are counted as direct bacterial counts (Wen and Hong-Bo, 2011).

Denaturing Gradient Gel Electrophoresis (DGGE) is a method used to separate DNA samples multiplied by PCR. DGGE has been used to resolve PCR-amplified regions of genes coding for 16S rRNA. This approach obtains profiles of microbial communities that can be used to identify temporal or spatial differences in community structure or to monitor shifts in the structure that occurs in response to environmental perturbations (Nakatsu, 2007). It is possible to infer the phylogeny of community members by DNA sequence analysis of amplified fragments after they have been excised (Wen and Hong-Bo, 2011).

Molecular biology technology and research strategy will promote the progress of environmental microbiology and technology, and is of great practical significance (Wen and Hong-Bo, 2011). Development of sophisticated genetic and imaging techniques has made it possible to characterize microorganisms and their spatial relationships to corrosion products and localized corrosion (Little *et al*., 2006).

1.2.7 INHIBITION OF CORROSION

In order to prevent and control biocorrosion in an industrial system, it is important to understand the operational conditions of the system. Hence, evaluation of the biological and abiotic parameters is essential when implementing prevention and control measures (Videla, 2002). In efforts to mitigate electrochemical corrosion, the primary strategy is to isolate the metal from corrosive agents (Oguzie, 2005; Lopez *et al.*, 2006). The inhibition of corrosion is a surface process which involves adsorption of the organic compounds on the metal surface. The efficiency of inhibition of organic compounds depends on the mode of interaction with the metal surface and molecular structure (Abiola and Oforka, 2004). MIC problems can be precluded by material selection however, economic and process constraints are often limiting factors (Jack and Westlake, 1995). Control of corrosion is important in limiting the dissolution of environmentally toxic metals from components (Ramesh and Rajeswari, 2004). From the industrial point of view, it should be more cost effective to adopt preventative measures rather than utilizing difficult and expensive remediation treatments due to failures. Methods commonly employed to prevent and control microbiological corrosion can be divided into several categories: (i) cleaning procedures; (ii) biocides; (iii) coatings; and (iv) cathodic protection (Videla, 2002). The use of corrosion inhibitors is usually the most appropriate way to achieve this objective (Oguzie, 2005).

Inorganic substances such as phosphates, chromate, dichromate, nitrite, nitrate and sulphide of alkali metals as well as salts of arsenic and cadmium have been found as effective inhibitors of metal corrosion. However, a major disadvantage is their toxicity and as such their use has come under severe criticism (Oguzie, 2005). The current trend for inhibitor usage is towards more environmentally friendly ‘green’ chemicals. Corrosion inhibitors reveal that most organic substances employed as corrosion inhibitors can adsorb on the metal surface through heteroatoms such as nitrogen, oxygen, sulfur and phosphorous, multiple bonds or aromatic rings and block the active sites, decreasing the corrosion rate. Moreover, nitrogen containing heterocyclic substances

such as triazole-type compounds are considered to be effective corrosion inhibitors of mild steel corrosion in acidic media (Ramesh and Rajeswari, 2004; Oguzie, 2005). Several nitrogen-containing organic compounds have been used as corrosion inhibitors for metal in acid environments (Abiola and Oforka, 2004). Benzoate compounds offer interesting possibilities for corrosion inhibition and are of particular interest because of their safe use and high solubility in water. The adsorption of the absorbate on the metal surface can markedly change the corrosion-resisting property of the metal and so the study of the relations between the adsorption and corrosion inhibition is of great importance (Rosliza *et al*., 2008).

The simple alteration of nutrient or physical parameters in an industrial system may promote a different, potentially more benign biofilm community. This concept has been tried in the field in a side stream test facility handling oil field brines. The presence of low levels of nitrate has long been known to control H_2S production in mixed microbial communities (Jack and Westlake, 1995). Surprisingly, bacteria can however, have a beneficial role in preventing corrosion. The bacterial protection of stainless steel can be the result of (i) production of inhibitors that are retained in the biofilm matrix; (ii) reduction of oxygen concentration at the metal surface; (iii) secretion of antimicrobial proteins; (iv) production of enzymes able to degrade the biofilm matrix (de Carvalho, 2007). The formation of biofilms on the surfaces of metals decrease the corrosion rate due to the consumption of the cathodic reaction, oxygen, therefore, rendering it unavailable for the corrosion reaction. However, corrosion inhibition by this mechanism requires a uniform biofilm on the entire metal surface with uniform microbial activity. This, however, renders difficult in nature due to the non-uniform biofilm formation and microbial activity (Lin and Ballim, 2012). Microorganisms can interfere with others while competing for an ecological niche. The relations between bacteria can be synergistic or antagonistic and bacterial interference can have a major role in the proliferation of infections (de Carvalho, 2007).

1.3 SCOPE OF THE PRESENT STUDY

Major industries locally and internationally are facing a dilemma due to the corrosion of pipes. This problem serves as a major financial strain to their businesses. Soils are known to promote corrosion of buried metallic structures like pipe lines for water, gas, oil, transmission lines, tanks, power cables, etc. (Lopez *et al.,* 2006). Factors attributed to this are the presence of moisture, air and electrolytes. Corrosion is also caused by the formation of differential aeration and concentration

cells due to soils of different compositions and textures present along the metal structures (Dayal *et al.,* 1988).

The deteriorative effect of biological elements is an important factor contributing to corrosion of outdoor metal facilities. The biological effect is usually brought about by biofilm formation, which is composed of different microorganisms and by excreted polymeric substances. The most severe biocorrosion failures occur in anaerobic environments, such as soils or under thick biofilms. The representative microorganisms of these environments are the SRB, which transform sulphate into sulphide, thus having a deteriorative effect on metal surfaces (Keresztes *et al.,* 2001).

Prevention of biocorrosion should be focused on inhibiting or reducing the development of biofilms. The traditional use of biocides is now being replaced with more effective and eco-friendly methods with more readily degradable compounds that are less toxic to the environment. Unfortunately, biocides are known to be toxic and this has a negative impact on the environment.

Another method for the mitigation and control of biocorrosion and SRB metabolic activities involves the addition of nutrients. The introduction of nutrients stimulates the activity of indigenous nitrate-reducing, sulphide-oxidising bacteria to lower sulphide concentrations that can outcompete SRB for energy sources and common electron donors (e.g. organic acids) by a strategy known as "biocompetitive exclusion" (Gevertz *et al*., 2000).

Thus, the current study focuses on the employment of this strategy to examine the rate of corrosion of mild steels and stainless steels under different environmental conditions. The metal coupons were tested in soil and marine conditions with additional nitrates. This study examines the effects of nutrients in reducing or inhibiting biofilm formation.

1.4 AIM OF THE STUDY

The aim of this study was to investigate the microbiologically-influenced corrosion activities of stainless and mild steels under different environmental conditions by studying the impact of additional source of nutrients in the corrosion process.

1.4.1 OBJECTIVES

1.4.1.1 To quantify, isolate and identify Microbiologically Influenced Corrosion (MIC) bacteria that stimulate corrosion of steel under different environmental conditions such as soil and marine conditions.

1.4.1.2 To study the microbial diversity in the biofilm of the corroded metals.

1.4.1.3 To measure the rate of corrosion and analyze the surface of corroded metals.

1.4.1.4 To investigate the correlation between biofilm formation and metal corrosion.

1.4.1.5 To study the impact of additional nutrients in biofilm formation and metal corrosion.

1.4.2 KEY QUESTIONS TO BE ANSWERED

1.4.2.1 What are the complex characteristics of the microbial communities found within the biofilm that play a role in MIC on steels?

1.4.2.2 Can the addition of nitrate or other nutrients induce a population shift to prevent or reduce metal corrosion?

1.4.2.3 If yes, what are the optimal environmental conditions for the prevention of MIC activities?

CHAPTER TWO
THE IMPACT OF ADDITIONAL NITROGEN SOURCES IN METAL CORROSION IN A STIMULATED SEAWATER/SEDIMENT SYSTEM

2.1 INTRODUCTION

Various attempts have been made to prevent/retard the destructive effect of corrosion on metals and alloys. Corrosion control can be achieved by many methods, of which the use of corrosion inhibition is one of the most effective alternatives (Blustein *et al*., 2005; Mousavi *et al*., 2011). Microbial growth and propagation on material surfaces can also be controlled by physical and chemical manipulations of the material and surrounding environment (Gu, 2003). Prevention against biodeterioration of metals includes surface engineering to reduce attachment by and susceptibility to microorganisms. As a control measure, lowering humidity, for example, has been shown to be an effective way to slow down the growth of microorganisms on surfaces in an enclosed environment and prevention against potential corrosion (Gu, 2003).

Bacteria are known to cause MIC and are generally found at the boundary layer between the pipe and interior deposits. It is often difficult to physically solve the problem with sterilizing chemicals alone (Momba *et al*., 2000). The development of biofilms can be controlled through the use of microbiocides, biodispersants, and by limiting nutrients (Chandy and Angles, 2001; Flemming and Ridgway, 2008; Simoes *et al*., 2010). The application of organic biocides is the most commonly adopted treatment in order to achieve microbiological control of microbial activities. Oxidizing and non-oxidizing microbiocides are widely used in contolling biofilms which cause corrosion and deterioration of a wide range of materials in industries (Cloete *et al*., 1998; Gu, 2003). The selection of biocides must carefully follow prescribed criteria and those chosen must be specifically relevant to the bacteria, design and operation of the system in order to kill the planktonic and sessile bacteria (biofilms) or retard bacterial growth (Sanders, 2003). Use of increased biocide dosage solely is generally useless, as they are only designed to suppress microbiological growths, not kill and eradicate them (Momba *et al*., 2000). Effective control is greatly dependent on frequency of addition, level of feed, and resistance of the microbial population to the biocide being used (Gu, 2003).

Although biocides targeting the SRB population are commonly used (Jayaraman *et al*., 1999b), they can however, cause the emergence of biocide-resistant SRB that do not readily penetrate biofilms within reservoirs or on the metal surfaces of industrial equipment (Gardner and Stewart, 2002). Biocides can also cause corrosion if applied in high concentrations (Zuo *et al*., 2004) or kill microbial community members that offer protection against corrosion (Potekhina *et al*., 1999).

Nitrates, benzoates, phosphates, chromates and borates are amongst many inhibitors that are widely employed to minimize corrosion of many metallic structures in various environments (Loto *et al*., 2011). A nitrate-based strategy is one of the most widespread of newly developing microbial control strategies, displaying much promise as a bacteriocide replacement (Thorstenson *et al*., 2002; Larsen, 2002). In laboratory tests, nitrate has been shown to suppress biogenic sulphide production by encouraging the activity of NRB and DNB (Mueller, 1996). The technology is based on the addition of low concentrations of a water-soluble nutrient solution that selectively stimulates the growth of indigenous nitrate-reducing, sulphide-oxidising bacteria, thereby inhibiting the detrimental SRB population that causes the generation of H_2S. This deliberate and controlled modification of the microbial populations has been termed biocompetitive exclusion (Sandbeck and Hitzman, 1995; Hitzman and Dennis, 1997; Gevertz *et al*., 2000; Videla and Herrera, 2005). The addition of nitrate and/or nitrite and the introduction of nitrate-reducing, sulphide-oxidizing bacteria (NR-SOB) such as *Thiobacillus denitrificans* inhibiting biogenic H_2S production, is a method to mitigate microbial activities of SRB (Jenneman *et al*., 1986; McInerney *et al*., 1993). To fully make use of this approach, extensive analysis of interactions between bacteria within the microbial community and interactions between certain bacteria and metal substratum has to be conducted (Zuo, 2007).

Field trials have been carried out on numerous oil reservoirs and water injection systems, (Thorstenson *et al*., 2002; Larsen, 2002) and results have shown significant reductions in MIC, SRB activity and hydrogen sulphide souring (Voordouw *et al*., 2002). Jenneman *et al.* (1986) and Jack *et al.* (1985) demonstrated that the addition of nitrate to sulfide-laden oil field brines could also be used to remove sulphides in these waters. McInerney *et al.* (1993) added ammonium nitrate to an injector at the Southeast Vasser Vertz Sand Unit in Oklahoma and reported a 40 to 60% reduction in sulphide at three adjacent producing wells that they attributed to the activity of indigenous NRB. Nitrite is another simple organic compound that has been used to inhibit SRB activity and reduce the souring potential of a system. Nitrite is a specific inhibitor of SRB (Sturman and Goeres, 1999).

In laboratory testing it is shown to chemically scavenge preexisting sulphide. Nitrite itself promotes corrosion at concentrations below 3.5 mM but serves as a corrosion inhibitor at concentrations of 10–20 mM (Hubert *et al*., 2005). Reinsel *et al.* (1996) observed lower concentrations of sulphides in the effluents of sandstone columns inoculated with produced water containing indigenous bacteria from two North Sea oil fields following injection with low concentrations of nitrate or nitrite (0.57 to 0.71 mM). However, they attributed the lower sulphide concentrations to nitrite inhibition of the indigenous SRB and not to oxidation of sulphides by indigenous NR-SOB. Sandbeck and Hitzman (1995) suggested that nitrate or nitrite in the presence of low concentrations of molybdate, an SRB inhibitor, is biocidal to SRB (Gevertz *et al*., 2000). Treatment of oil and gas wells with nitrite resulted in significant and rapid reductions in H_2S, as well as the virtual elimination of SRB in water samples. Beneficial effects of the nitrite souring control technology have been observed for three to seven months after the initial treatment (Sturman and Goeres, 1999).

Nitrite is one component of biocompetitive exclusion technology which uses a defined inorganic bacterial nutrient supplement to encourage a beneficial microbial population to inhibit sulphate reduction (Hitzman and Dennis, 1998). Nitrite-based control strategies have the potential for further development and application protocols for specific systems need to be developed so that simple dosing guidelines can be established (Sanders, 2003). Although a nitrate treatment can be very effective, it may not control biofouling. Corrosion and souring control by nitrite thus requires a dose that is sufficiently high to directly inhibit SRB activity and prevent souring and corrosion problems (Hubert *et al*., 2005). There is also evidence that nitrate utilizing bacteria (NUB) can contribute to MIC, so that the negative aspects of this type of treatment have to be assessed to ensure that unwanted side effects do not occur (Voordouw *et al*., 2002).

Several mechanisms may contribute to the containment of biodeterioration by nitrate or nitrite addition:

- Competition between SRB and heterotrophic nitrate- or nitrite-reducing bacteria (hNRB) for available organic electron donors may result in the competitive exclusion of SRB. Similarly, some SRB may switch their energy metabolism to reduce nitrate or nitrite instead of sulphate, i.e. SRB may become hNRB (Eckford and Fedorak 2002).
- NR-SOB use nitrate or nitrite to re-oxidize produced sulphide to elemental sulphur and sulphate, creating a sulphur cycle involving NR-SOB and SRB that results in net sulphide

removal when insufficient organic electron donors are present to reduce all added nitrate (Hubert *et al*., 2003).

- Nitrite (but not nitrate) inhibits the reduction of sulphite to sulphide by the enzyme dissimilatory sulphite reductase, the terminal enzymatic step in the sulphate reduction reaction pathway of SRB (Nemati *et al*., 2001a; Greene *et al.*, 2006).

It is likely that all these mechanisms operate differently in different environments however, the interactions between NUB, SRB and environmental conditions are still not well understood (Nemati *et al.,* 2001b). The impact of additional nitrate/nitrite treatments and the long-term consequences for MIC are poorly understood. Field investigations on NO^{3-}/NO^{2-} efficacy for souring and corrosion control have previously focused on planktonic communities, cell suspensions, or debris instead of substrate-attached intact biofilms. Local concentrations of NO^{3-} and its reduction products inside the biofilms are unknown but are essential for evaluating the effectiveness of the treatment, as is the *in situ* impact of NO^{3-} on a complex bacterial community (Schwermer *et al*., 2008).

This chapter describes the impact and effectiveness of inorganic salts such as sodium nitrate and ammonium nitrate in a simulated water/sediment system. The steel samples were analyzed to observe whether the nitrates served as a nutrient to the denitrifying bacteria, causing them to flourish and deny essential nutrients for SRB metabolic action and ultimately result in the inhibition of corrosion of the steel samples.

2.2 MATERIALS AND METHODS

2.2.1 SAMPLE COLLECTION AND PREPARATION

Seawater was collected (from the Durban beachfront, KwaZulu-Natal) and tested for temperature, pH and salinity at the time of collection. The salinity of seawater was determined by measuring the total dissolved salts as described by McCauley *et al*. (2003). This was accomplished by evaporating a known volume of water to dryness and measuring the remaining solid residue. The sediment was collected at the water/sediment interface. Metal coupons used in this study were obtained from the Academic Instrumentation Unit (University of KwaZulu-Natal, Westville). The coupons were made of mild steel and commercial grade 304 stainless steel, with dimensions of 2.5×2.5 cm. Coupons were 1.2 mm thick with a 2 mm drilled mounting hole. The coupons were degreased with acetone.

The degreased sides of the coupons were polished with fine polishing paper and rinsed with distilled water. Prior to experiments, the coupons were weighed to the sensitivity level of 0.0001 g using an analytical balance (Sartorius Basic).

2.2.2 CONSTRUCTION OF THE SEAWATER/SEDIMENT SYSTEM AND DETERMINATION OF CORROSION BY WEIGHT LOSS MEASUREMENTS

Experiments with a water/sediment system using sediment and seawater were constructed (Orfei *et al.,* 2006). Four 3 L catering jars were filled with 1 L of seawater and 2 kg of sea sand and subjected to different conditions. One jar contained autoclaved sea water and sea sand and was employed as the control. The second jar was filled with non-autoclaved sea water and sea sand. The final two jars contained additional sources of nitrates (20 ml with a final concentration of 5 mM) such as sodium nitrate or ammonium nitrate (added at each sampling time) in an attempt to inhibit or reduce the rates of corrosion on steel. The concentration of the nitrate supplements (5 mM) was according to the study conducted by Aung and Tan, 2004. The coupons were suspended into the jars in a vertical position. Corrosion was enhanced by the slow addition of 20 ml of distilled water at different time intervals of the experiment (Aung and Tan, 2004). At the end of the experiment, the corrosion products on the metal coupons (duplicate) were removed from the exposed metal surfaces by complete immersion for 90 secs in 20 % hydrochloric acid under constant agitation, followed by a rinse in distilled water and air drying (Ryhl-Svendsen, 2008). Thereafter, the coupons were cleaned under a stream of tap water and scrubbed vigorously with a rubber stopper. After cleaning, the metal coupons were immediately wiped with paper towel and dried in an oven at 80 °C for 10 mins. After cooling, the mass loss (mg) was measured (Zuo *et al.,* 2004). The weight loss was normalized to the original weight.

2.2.3 ENUMERATION OF THE TOTAL HETEROTROPHIC AND SULPHATE-REDUCING BACTERIAL POPULATION PRESENT ON THE SURFACE OF THE COUPONS

Sampling for microbiological analyses was performed by swabbing duplicate coupons over a period of time. The cotton swabs (25 mm Gamma sterilized) were immersed into different solutions to determine the total heterotrophic bacteria and SRB populations. Most probable number was determined using the Most Probable Number Calculator© Version 4.04 (Klee, 1996). The number of bacteria was quantified using the three-tube Most Probable Number (MPN) technique. Solutions

containing 2 % NaCl and 0.85 % NaCl, as well as Postgate C solutions (Appendix I) were used for total heterotrophic and sulphate-reducing bacterial enumeration respectively. These solutions were vortexed at full speed (Model K-550-6E, 220 V, 50 Hz and 0.5 Amp) in order to disperse the cells from the seawater/sediment system. Thereafter, serial dilutions of these suspensions were prepared in distilled water. One millilitre of each dilution was inoculated into nutrient broth and Postgate B medium (Appendix I) respectively. The tubes were incubated at 30 °C for 48 hrs (total heterotrophic bacterial enumeration) and for 7-10 days (SRB enumeration). The tubes exhibiting a black precipitate were scored positive for SRB growth and scored positive for total heterotrophic bacteria by observing growth and turbidity in the nutrient broth (Lutterbach and de Franca, 1997; Lopes *et al*., 2006**)**.

2.2.4 ANALYSIS OF THE CARBOHYDRATE CONTENTS OF THE BIOFILM DEVELOPED ON THE COUPON SURFACE

The carbohydrate contents of the biofilm developed on the coupon surface were calculated using standard D-glucose curves (Beech *et al*., 2000**)**. At each sampling time, the biofilm was scraped off the metal coupon and suspended in 2 ml distilled water for carbohydrate and protein estimation. The suspension was vigorously vortexed and 100 µl of sample was analyzed using the DNS assay for total reducing sugars. The absorbance was measured at an optical density of 540 nm against a reagent blank.

2.2.5 ESTIMATION OF THE PROTEIN CONTENTS OF THE BIOFILM DEVELOPED ON THE COUPON SURFACE

Protein measurements were performed using the Bradford assay (1976). Two milliliters of Bradford reagent were added to 100 µl of sample mentioned in Section 2.2.4 and vortexed. Each sample was allowed to incubate at room temperature for 15 min and no longer than one hour before being measured at an optical density of 595 nm. The sample concentration was calculated using a standard bovine serum albumin curve (Beech *et al*., 2000**)**.

2.2.6 MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS

Mild steel coupons were subjected to chemical fixation, a modified method of Faimali *et al*. (2004). The coupons were immersed in 2.5 % glutaraldehyde (which immobilized the biofilm on the coupon surface) and phosphate buffer for 5 min. Thereafter, the coupons were treated to a series of alcohol for 5 mins each. The final step was immersion for 5 mins in Hexamethyldisilazane (Murga *et al*., 2001). The coupons were then coated with gold using a Polaron SC500 Gold Sputter Coater for 2 mins at 0.1 Torr and viewed using the SEM Leo 1450. Mild steel coupons were also examined using Energy-Dispersive X-Ray analysis using the Jeol JSM 6100 SEM equipped with a Bruker EDX detector and a Nikon Stereo Microscope after the biofilm and corrosion products were removed (Chongdar *et al*., 2005).

2.2.7 STATISTICAL ANALYSIS

Data were analyzed using SPSS for windows (version PASW18, SPSS Inc., Chicago, IL). A one-way ANOVA was used to test mean differences in weight losses of the metal coupons over time. A p-value of <0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 PHYSICAL CHARACTERIZATION OF THE SAMPLING SITE

Table 2.1 shows the physical characteristics of sea sand and sea water. It was observed that the sea water was acidic (pH 5.71) and the sea sand had a high percentage of water content (8.04 %). The temperature of the sea water was 24 °C at the time of collection and the percentage of salinity was 4.1 % of salt solution.

Table 2.1: The physical characteristics of the sampling site

	SEA SAND WITH SEA WATER
pH	5.71
PERCENTAGE WATER CONTENT	8.04 %
PERCENTAGE DRY MASS	91.96 %
TEMPERATURE OF SEA WATER	24 ˚C
SALINITY	4.1 % salt solution

2.3.2. WEIGHT LOSS MEASUREMENTS OF STAINLESS STEEL COUPONS IMMERSED IN THE SEAWATER/SEDIMENT SYSTEM

In this study the preliminary investigation of the corrosion process was conducted with commercial grade 304 stainless steel coupons in a stimulated seawater/sediment system. The results of weight loss of stainless steel coupons are shown in Figure 2.1. The stainless steel coupons demonstrated a decrease in weight loss measurements at week 12. An increase in weight loss was observed at 16 and 20 weeks. An increase in weight loss was observed in samples supplemented with ammonium nitrate (0.135 mg/g) compared to the non-autoclaved sample (0.075 mg/g) and the autoclaved control (0.075 mg/g) at 20 weeks. However, there was no significant difference between the treatments when compared to the autoclaved control and the non-autoclaved sample. In this study there was a decrease in weight loss measurements of the coupons observed over time. Microscopic analyses on the surface of the stainless steel coupons revealed minimal signs of corrosion (mg/g of weight loss) after 10 months of exposure to the stimulated seawater/sediment system. After 16 months of exposure, pitting on the surface of the coupons was visible however, due to the time factor of measuring the weight loss of the stainless steel coupons, the study focus shifted to the corrosion of mild steel coupons instead.

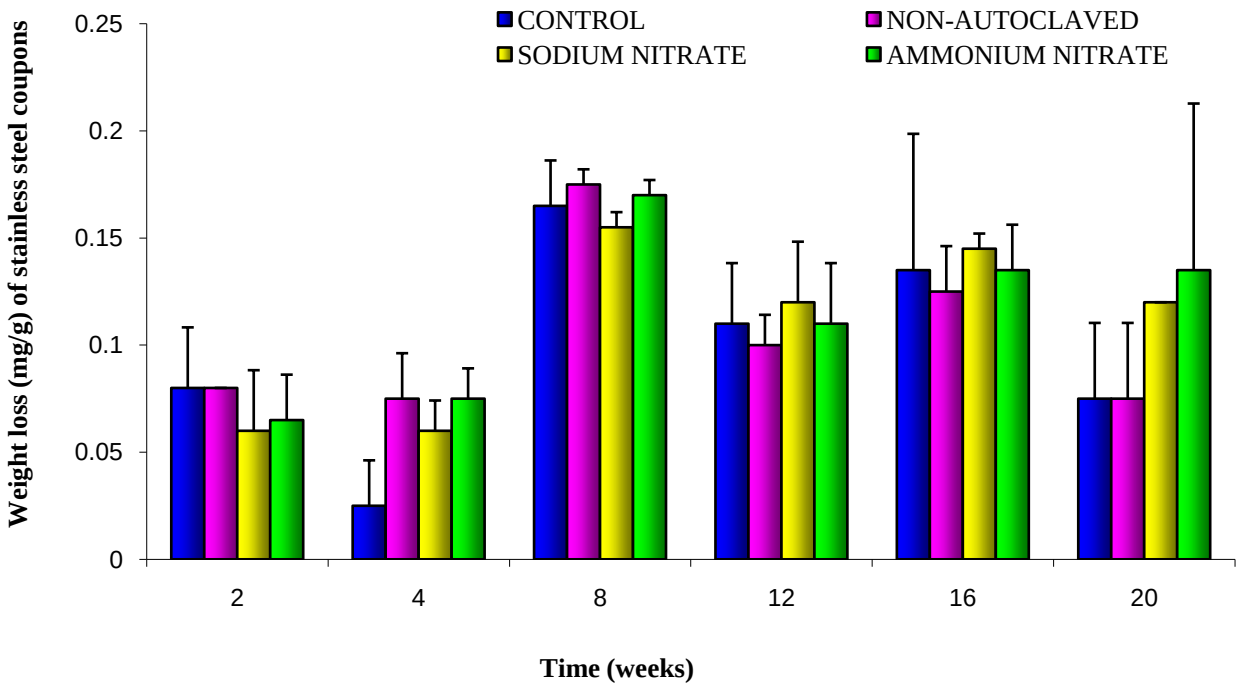

Figure 2.1: Weight loss of stainless steel coupons in the seawater/sediment system for a 20 week period

2.3.3 WEIGHT LOSS MEASUREMENTS OF MILD STEEL COUPONS IMMERSED IN THE SEAWATER/SEDIMENT SYSTEM

The weight loss measurements of mild steel coupons immersed in a stimulated seawater/sediment system are shown in Figure 2.2. Weight loss of mild steel coupons increased with the incubation period under all experimental conditions. The coupons in the autoclaved and non-autoclaved systems demonstrated similar weight losses from 1.78 mg/g at week 2 to 6.69 mg/g at week 12 and 2.08 mg/g at week 2 to 6.83 mg/g at week 12 respectively. A significant increase ($p < 0.05$) in weight loss of the mild steel coupons was observed in the sample supplemented with ammonium nitrate throughout the 20 week study period (6.13 mg/g at week 2 to 20.97 mg/g at week 20) compared to the sterilized control and those without nitrate supplements. There is no significant difference ($p > 0.05$) when different nitrate sources were added to the stimulated system. Reduced weight loss (7.09 mg/g) of the coupons was observed in the non-autoclaved sample at week 20 as compared to the other samples in the stimulated seawater/sediment system.

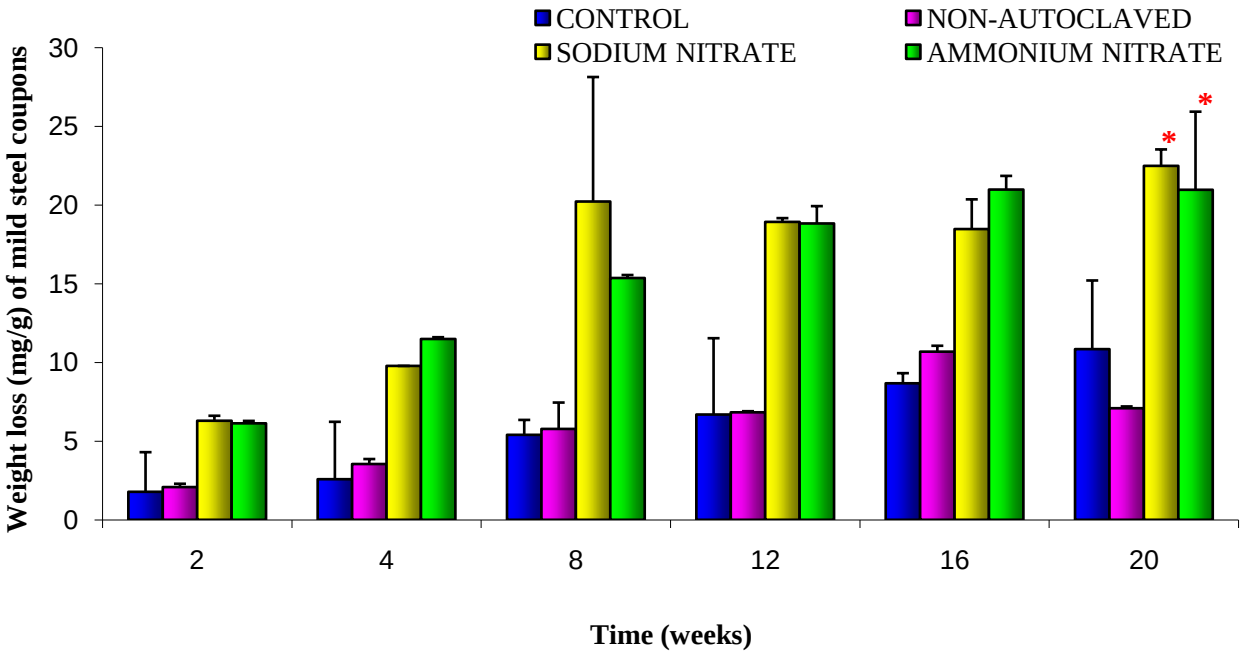

Figure 2.2: Weight loss of mild steel coupons in the seawater/sediment system for a 20 week period (* - significant difference $p < 0.05$)

2.3.4 ENUMERATION OF TOTAL HETEROTROPHIC BACTERIA AND SULPHATE REDUCING BACTERIAL POPULATIONS ON THE METAL SURFACE

The total heterotrophic and sulphate-reducing bacterial populations extracted from the biofilm samples on the surface of the mild steel coupons were enumerated using the MPN technique. This was expressed as the MPN/ml based on the results obtained for MPN corrected for bias (Salama) using the Most Probable Number Calculator.

The results of the enumeration of the total heterotrophic bacterial population extracted from the surface of the mild steel coupons immersed in the stimulated seawater/sediment system are shown in Figure 2.3. An increase in the total heterotrophic bacterial growth was observed in the sample supplemented with sodium nitrate at week 4 (8.877 MPN/ml) as compared to the non-autoclaved sample (7.417 MPN/ml) and the autoclaved control (5.515 MPN/ml). During the initial stages, reduced population growth was observed in the sample supplemented with ammonium nitrate (0 MPN/ml at week 2 to 0.052 MPN/ml at week 8), but thereafter, an increase in growth was observed at week 12 (7.477 MPN/ml). Increased total heterotrophic bacterial growth was observed in the

non-autoclaved sample (7.394 MPN/ml) and in the sample stimulated with ammonium nitrate (3.599 MPN/ml) at week 20. However, the population growth of the total heterotrophic bacteria present in all samples was not significantly different ($p > 0.05$).

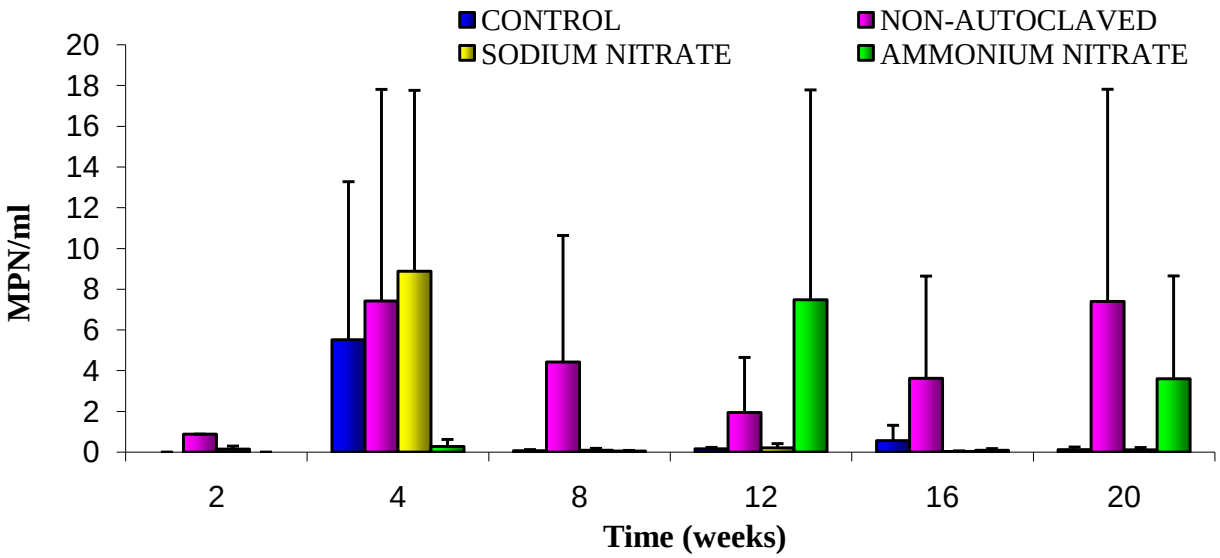

Figure 2.3: Enumeration of total heterotrophic microorganisms on mild steel coupons in the seawater/sediment system over a period of 20 weeks

The enumeration of the population growth of sulphate-reducing bacteria extracted from the surface of the mild steel coupons immersed in the stimulated seawater/sediment system are shown in Figure 2.4. A significant increase in the population growth of anaerobic bacteria was observed after only two weeks of incubation in both the nitrate treated systems (34.52 MPN/ml and 6.13 MPN/ml for sodium nitrate and ammonium nitrate treated systems respectively) and the non-autoclaved sample (16.207 MPN/ml) as compared to the autoclaved control (0.198 MPN/ml). A decrease in anaerobic bacterial growth was observed in all samples except for the non-autoclaved sample at week 8 (10.585 MPN/ml). At week 12 to week 20, reduced growth of the anaerobic population was observed in all samples. A decrease in population growth was observed in the non-autoclaved sample (0.069 MPN/ml) and the nitrate treated samples (0.030 MPN/ml and 0.129 MPN/ml for sodium nitrate and ammonium nitrate treated systems, respectively) at week 20.

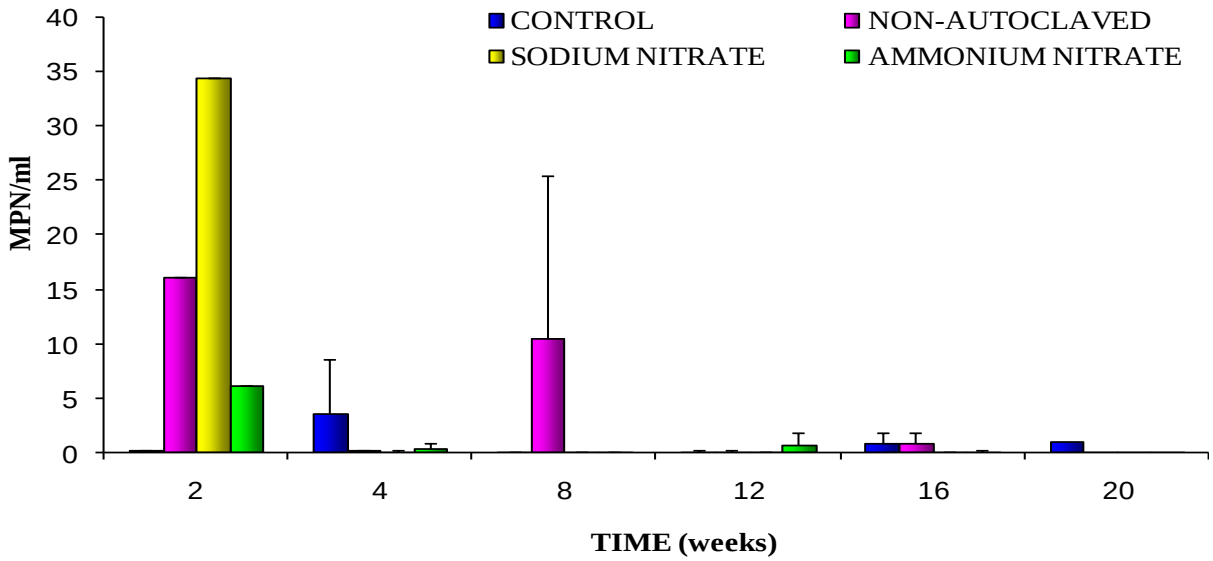

Figure 2.4: Enumeration of sulphate-reducing microorganisms on mild steel coupons in the seawater/sediment system over a period of 20 weeks

2.3.5 THE EVALUATION OF THE CARBOHYDRATE AND PROTEIN CONCENTRATIONS PRESENT IN THE BIOFILM SAMPLES ON MILD STEEL SURFACE

Total protein concentration in the biofilm samples extracted from mild steel coupons immersed in the stimulated seawater/sediment system is shown in Table 2.2. During the initial stages of the experiment, protein content was not detectable in any of the samples in the stimulated systems. However, a significant ($p < 0.05$) increase in protein concentration was only observed in the non-autoclaved sample at week 12 and week 16 (0.078 mg/ml and 0.688 mg/ml, respectively) as compared to the autoclaved control. In the nitrate treated systems, no protein content was detected except for the sample supplemented with ammonium nitrate at week 20 (0.148 mg/ml). At the end of the experiment, a significant ($p<0.05$) increase in the concentration of protein was observed in the non-autoclaved sample (0.208 mg/ml) as compared to the autoclaved sample (0.146 mg/ml).

Table 2.2: Total protein concentration (mg/ml) in biofilm samples extracted from mild steel coupons over a period of 20 weeks

Incubation time	4wks	8wks	12wks	16wks	20wks
	Total Protein Concentration (mg/ml)				
CONTROL	0	0	0	0	0.146
NON-AUTOCLAVED	0	0	0.078	0.688	0.208
SODIUM NITRATE	0	0	0	0	0
AMMONIUM NITRATE	0	0	0	0	0.148

Total carbohydrate concentration of the biofilm formed on the surface of the mild steel coupons immersed in the stimulated seawater/sediment system is shown in Table 2.3. The results demonstrate a similar trend as the protein concentration of biofilms as shown above. Carbohydrate concentrations were only detected in the autoclaved control and the non-autoclaved sample (2.983 mg/ml and 3.960 mg/ml respectively) at week 4 and not in the nitrate treated systems. The concentration of carbohydrates gradually increased significantly ($p<0.05$) in the sample stimulated with sodium nitrate until 12 weeks (0 mg/ml at week 4 to 3.951 mg/ml at week 12). There was a significant increase in concentration of carbohydrates observed in the sample with additional ammonium nitrate until 16 weeks (0 mg/ml at week 4 to 3.516 mg/ml at week 16) and thereafter a decrease in concentration was observed (2.513 mg/ml at week 20).

Table 2.3: Total carbohydrate concentration (mg/ml) in biofilm samples extracted from mild steel coupons over a period of 20 weeks

Incubation time	4wks	8wks	12wks	16wks	20wks
	Total Carbohydrate Concentration (mg/ml)				
CONTROL	2.983	4.266	4.174	4.126	2.529
NON-AUTOCLAVED	3.960	2.502	3.375	1.066	2.486
SODIUM NITRATE	0	1.179	3.951	2.926	3.114
AMMONIUM NITRATE	0	2.059	3.161	3.516	2.513

2.3.6 SCANNING ELECTRON MICROSCOPY AND ELECTRON DIFFRACTION X-RAY ANALYSES OF CORRODED MILD STEEL COUPONS EXTRACTED FROM THE SEAWATER/SEDIMENT SYSTEM

2.3.6.1 SCANNING ELECTRON MICROSCOPY (SEM)

Mild steel coupons were extracted from the seawater/sediment system after 4, 8, 12, 16 and 20 weeks of incubation and subjected to SEM. Figure 2.5 shows an SEM image of an untreated mild steel coupon that has not been exposed to the stimulated seawater/sediment system. There are clear striations on the coupon surface that might be due to the polishing of the coupons prior to immersion into the system. SEM observations of the mild steel coupons immersed in the stimulated seawater/sediment system after four weeks of incubation are shown in Figure 2.6. The autoclaved control (Figure 2.6a) displayed microbial activity and the formation of deep cracks is visible as compared to the untreated mild steel coupon (Figure 2.5). The deep cracks observed on the surface of the coupons suggest that the corrosion process occurs even in the autoclaved control sample in the stimulated system. However, the microbial activity observed on the coupon surface was reduced as compared to the non-autoclaved sample (Figure 2.6b) and the nitrate-treated samples. Colonization of the mild steel coupons by microorganisms occurred relatively fast from the beginning of the experiment. This was observed by the presence of biofilm formation and microbial adherence (arrows in Figures 2.6c and d) on the mild steel surface after four weeks of incubation with the addition of sodium nitrate and ammonium nitrate respectively.

Figure 2.7 shows the SEM observation of the mild steel coupons after 12 weeks of incubation in the stimulated seawater/sediment system. Deep cracks were observed on the surface of the mild steel coupons in the autoclaved stimulated system (Figure 2.7a) similar to that observed after four weeks of incubation in the autoclaved sample (Figure 2.6a), but to a greater extent with longer exposure to the stimulated system. There are more microorganisms found in the mild steel coupons at week 12 compared with the coupons at week 4. The non-autoclaved sample displayed microbial cluster formation (arrow in Figure 2.7b) on the coupon surface, which is likely due to the natural response of the microbes that tend to aggregate in order to reduce the total surface area in contact with the environment. More web-like, needle-like structures were observed in the nitrate treated systems (arrows in Figures 2.7 c and d) as compared to the autoclaved control and those coupons in week 4. This structure was observed to compose bacterial cells, EPS and some water channels. More

bacterial cells were also observed to be embedded within the biofilm with the progress of the incubation time from four weeks to 12 weeks. There was much more product formation related to the corrosion process observed on the surface of the metal coupons in the non-autoclaved sample and the sample with additional nitrates.

SEM observations of the mild steel coupons extracted from the seawater/sediment system after 20 weeks of incubation are shown in Figure 2.8. The autoclaved sample (Figure 2.8a) showed some product formation related to the corrosion process on the surface of the coupon. Microbial colonization and biofilm formation was observed on the surface of the coupons in the non-autoclaved sample (arrow in Figure 2.8b) and the samples with additional nitrates (Figure 2.8 c and d) throughout the study as seen after 20 weeks of incubation. In the sample supplemented with sodium nitrate, microbial activity and needle-like product formation was observed on the coupon surface (arrow in Figure 2.8c). Increased microbial activity was observed on the surface of the mild steel coupon in the sample stimulated with ammonium nitrate (Figure 2.8d).

2.3.6.2 ENERGY DISPERSIVE X-RAY ANALYSIS

Mild steel coupons were extracted from the stimulated seawater/sediment system after 4, 8, 12, 16 and 20 weeks of incubation and analyzed for its elemental compositions using an SEM equipped with a Bruker EDX detector. The untreated mild steel coupons mainly comprised of iron (69.66 wt. %), oxygen (30.08 wt. %) and manganese (0.106 wt. %) (Figure 2.9 and Table 2.4) before they were immersed in the stimulated seawater/sediment system. Trace amounts of calcium, aluminium and silicon were also present on the coupon surface (0.016 wt. %, 0.07 wt. % and 0.02 wt. % respectively). EDX analyses of treated mild steel coupons revealed that increases in carbon, magnesium, phosphorus and sodium contents, which are important for any biological organisms, in addition to the main components of iron and oxygen were evident as shown in Figures 2.10 b - d. Other trace metals were also detected. In the sample supplemented with sodium nitrate (Figure 2.10 c(i) and c(ii)) after four weeks of incubation, higher concentrations of carbon (4.73 wt. %) and calcium (12.78 wt. %) were present as compared to the other samples in the stimulated seawater/sediment system.

Figure 2.5: Scanning electron image of an untreated mild steel coupon

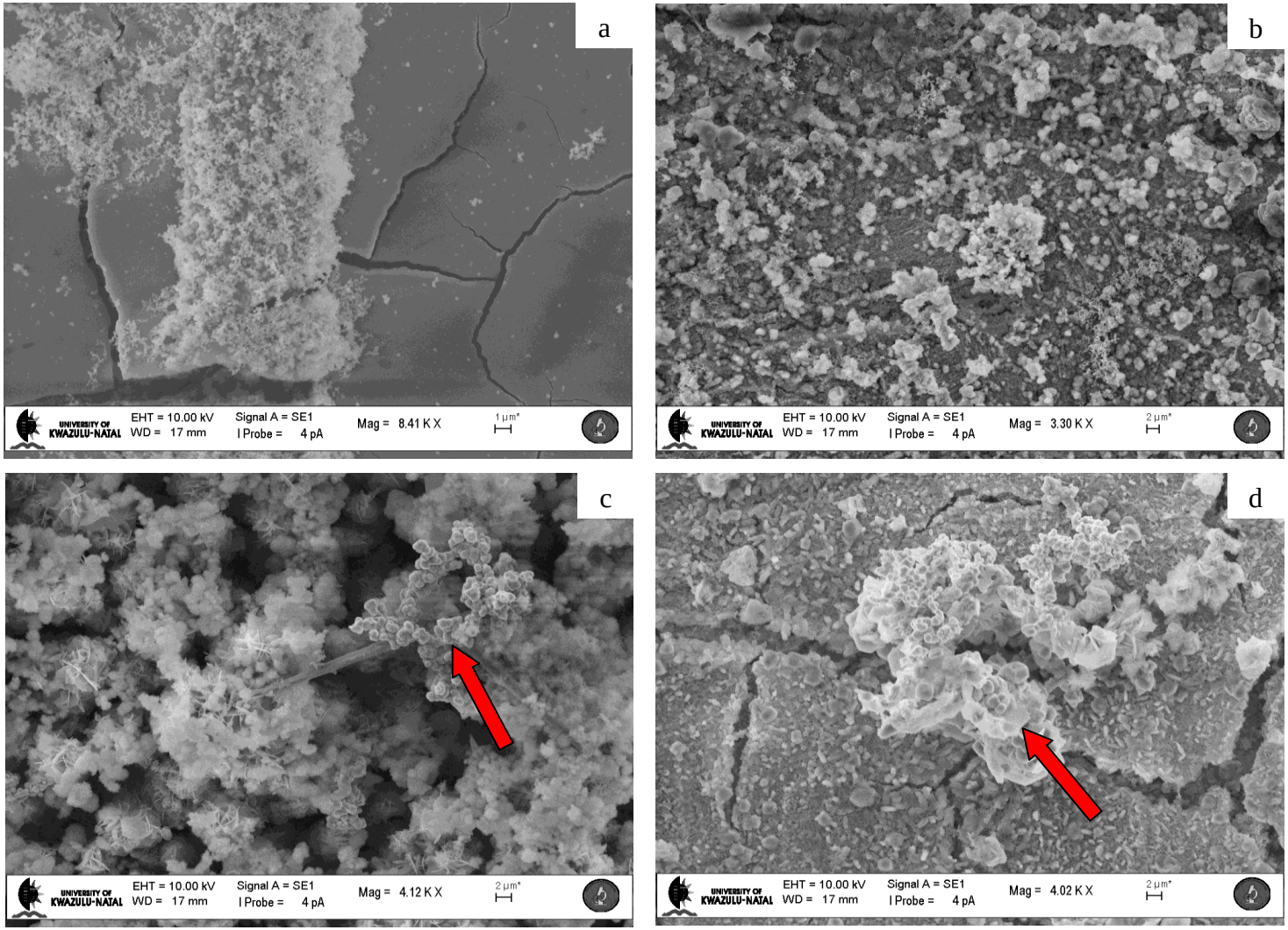

Figure 2.6: Scanning electron image of mild steel in seawater/sediment system after 4 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

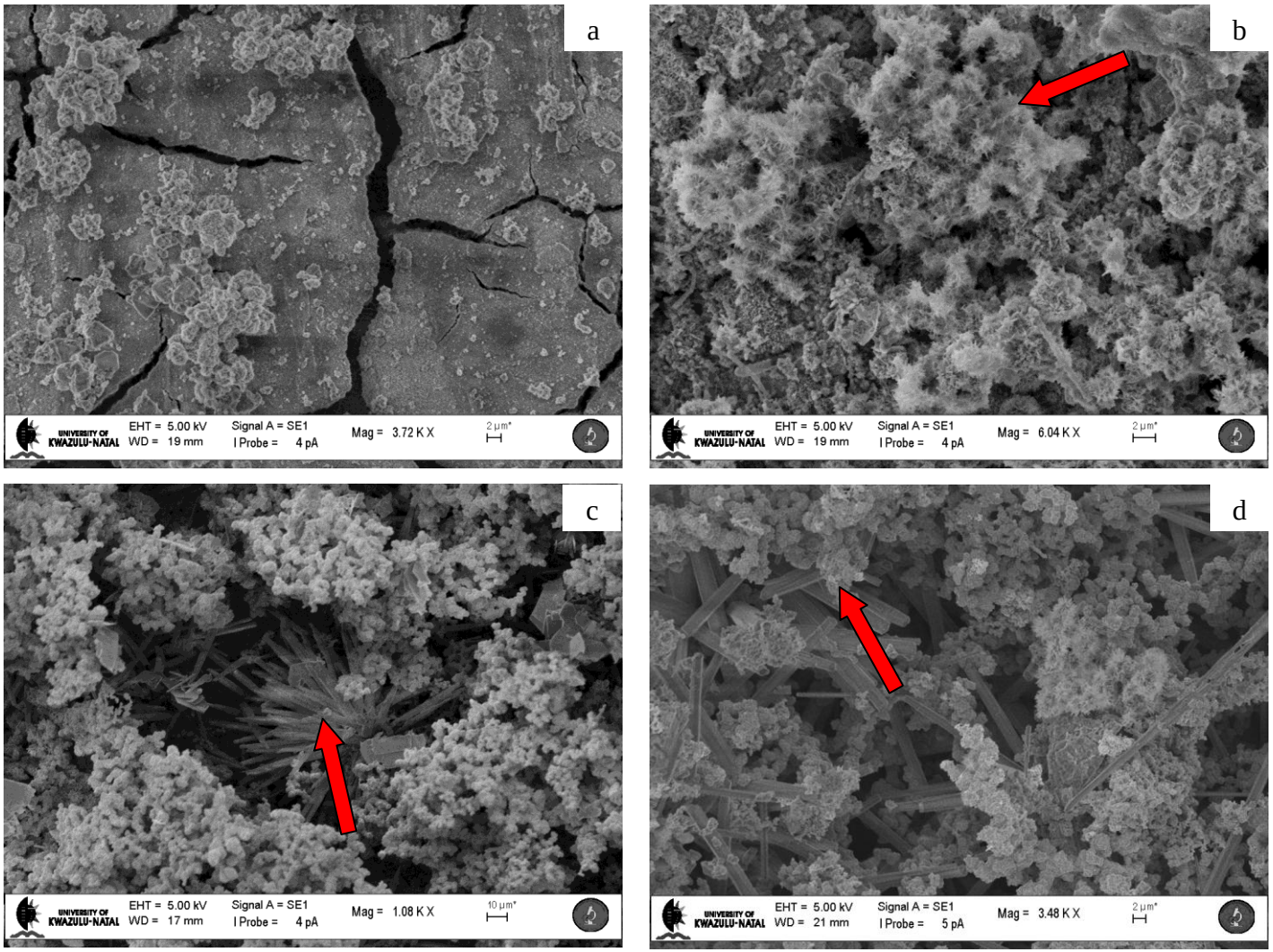

Figure 2.7: Scanning electron image of mild steel in seawater/sediment system after 12 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

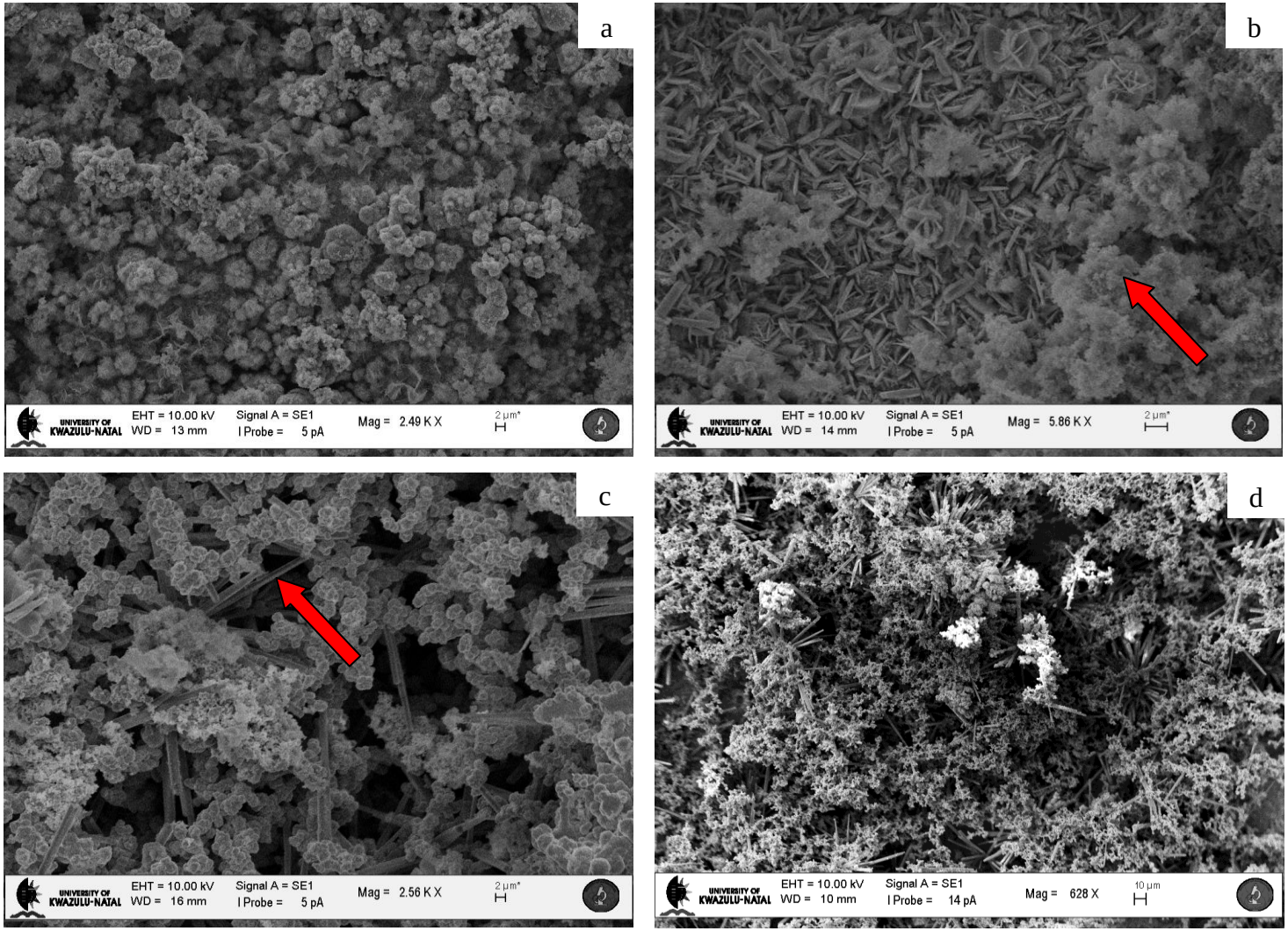

Figure 2.8: Scanning electron image of mild steel in seawater/sediment system after 20 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

Different elemental compositions of the mild steel coupons immersed in a stimulated seawater/sediment system over a 20 week study period are shown in Table 2.4. EDX analyses of the non-autoclaved sample after 12, 16 and 20 weeks detected greater concentrations of carbon (4.47 wt. %, 4.26 wt. % and 4.55 wt. % respectively) and sulphur (3.733 wt. %, 1.56 wt. % and 3.726 wt. % respectively). High concentrations of sulfur (3.733 wt. %) and iron (52.27 wt. %), which is characteristic of iron sulphide production by the reaction of the sulphides generated by the SRB, were detected in the non-autoclaved sample after 12 weeks of incubation. Increased concentrations of calcium (14.05 wt. %), carbon (4.26 wt. %) and oxygen (30.19 wt. %) were detected at week 16, indicating the formation of calcium carbonate precipitates. Increased concentrations of carbon (4.55 wt. %) and sulfur (3.726 wt. %) were detected in the non-autoclaved sample (Table 2.4) after 20 weeks of incubation. Higher concentration of phosphorous was detected in the autoclaved control (4.036 wt. %) and in the sample with additional ammonium nitrate (4.35 wt. %) as seen in Table 2.4. Greater concentration of magnesium (4.33 wt. %) was detected in the sample supplemented with ammonium nitrate.

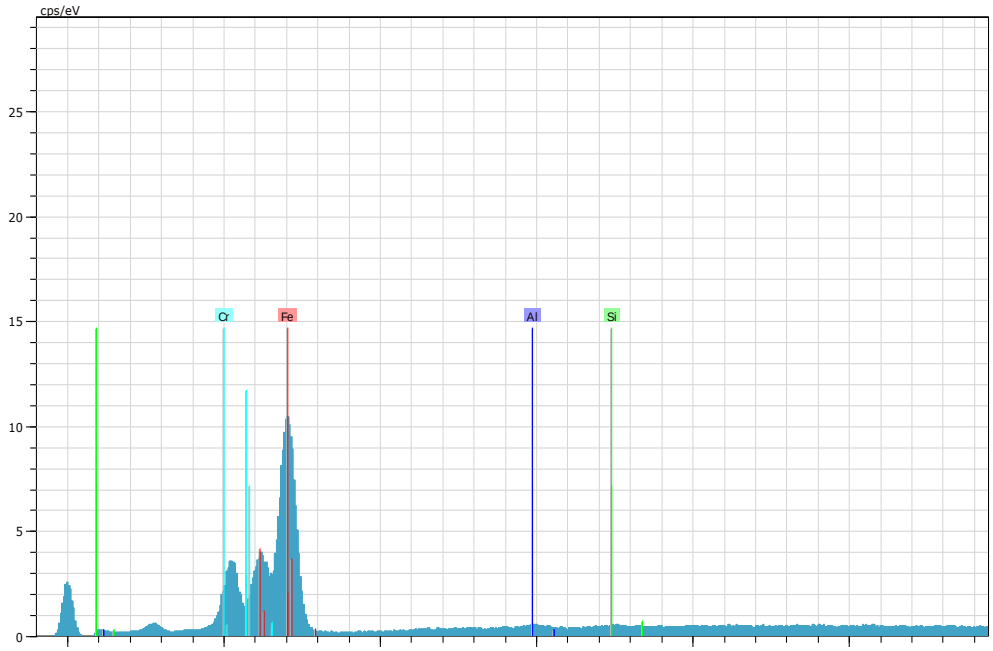

Figure 2.9: EDX spectra showing different elemental composition on the surface of an untreated mild steel coupon

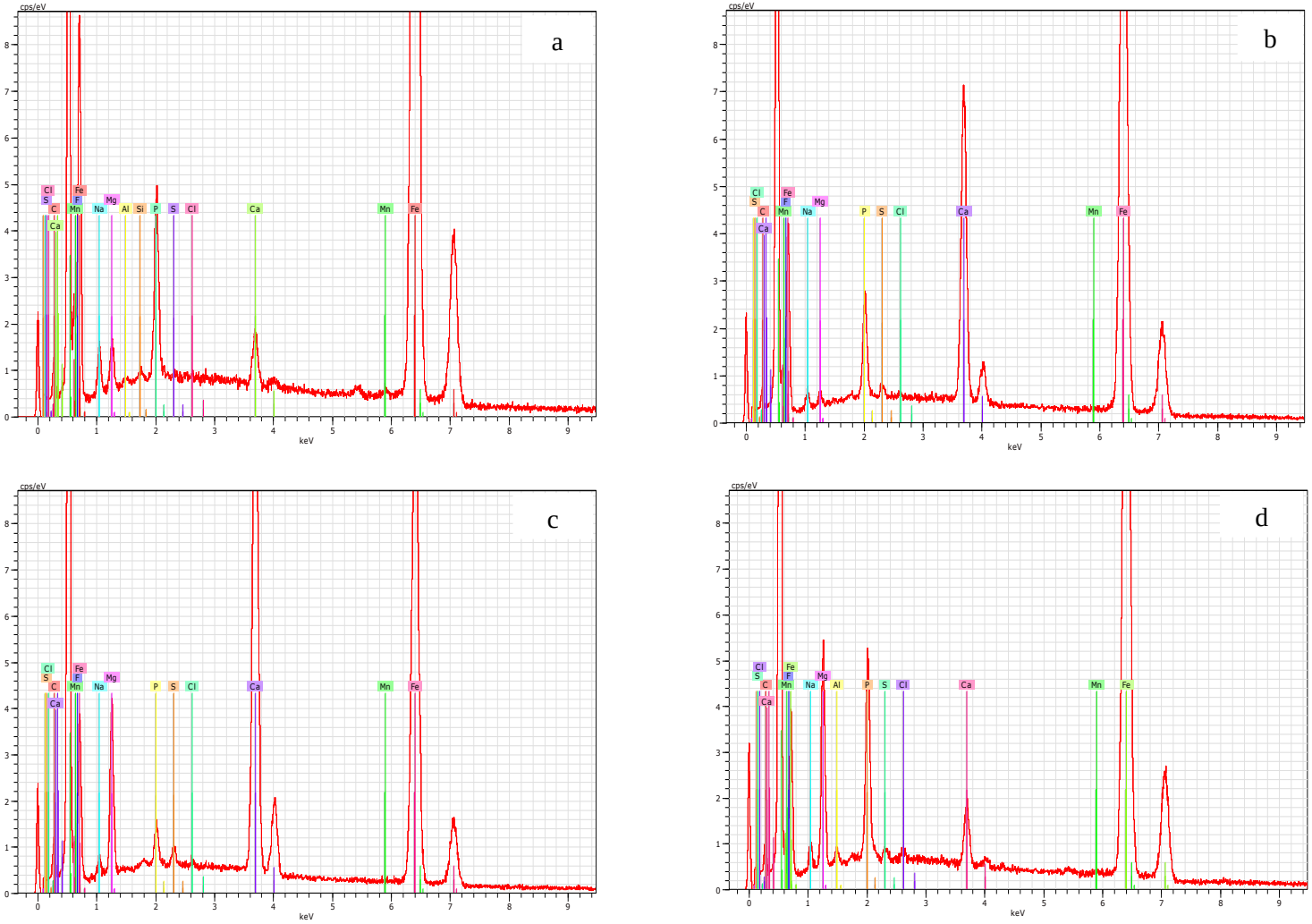

Figure 2.10: EDX spectra showing different elemental composition on the surface of the mild steel coupons after 4 weeks of incubation.

(a) Autoclaved control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

Table 2.4: Weight percentage values of different elemental compositions found on an untreated coupon, the autoclaved control and the non-autoclaved sample using the Energy Dispersive X-Ray Analysis

TIME (WEEKS)	C	F	Mn	Na	Mg	Al	Si	P	Ca	Fe	O	S
UNTREATED COUPON												
0	0	0	0.106	0	0	0.07	0.02	0	0.016	69.66	30.08	0.01
AUTOCLAVED CONTROL												
4	3.37	0.19	0.39	1.59	3.123	0.06	0.143	3.21	1.64	55.08	30.99	0
8	0	3.17	0	0.63	0.5	0.573	1.29	0.606	0.936	61.89	30.31	0.01
12	2.056	0	0	1.853	0.716	0.336	0.426	2.44	0.183	60.06	30.95	0.403
16	2.21	0	0.156	2.103	1.156	0.04	0.066	3	0.39	59.64	30.75	0.056
20	2.406	0	0.13	3.723	0.58	0.076	0.04	4.036	0.183	57.95	30.79	0.056
NON-AUTOCLAVED CONTROL												
4	3.336	0.093	0.203	1.593	0.3	0.746	0.003	1.986	2.963	58.83	29.47	0.19
8	0	0	0	0.656	0.866	0	0	2.466	0.313	64.04	31.41	0
12	4.47	0	0	2.136	1.513	0.356	0.266	2.866	0.586	52.27	31.76	3.733
16	4.26	0	0.116	0.66	3.023	0.026	0.276	1.133	14.05	44.69	30.19	1.56
20	4.55	0	0.07	0.963	1.75	0.013	0.11	1.183	8.023	48.86	30.75	3.726
SODIUM NITRATE												
4	4.73	0	0.236	1.323	4.193	0	0.006	1.446	12.78	45.42	29.53	0.21
8	0	0	0	0	0.176	0	0	0	22.04	61.65	29.22	0
12	3.79	0	0	0.86	1.673	0.136	0.096	1.556	0.98	60.39	29.84	0.013
16	1.79	0	0.163	0.303	1.013	0	0.043	0.456	2.266	64.10	29.83	0.026
20	3.103	0	0.363	1.223	2.62	0.173	0.166	1.956	4.033	55.78	30.3	0.016
AMMONIUM NITRATE												
4	3.60	0	0.29	1.81	2.90	0.21	0	2.49	0.72	57.2	30.4	0.14
8	0	0	0.08	0	0.39	0	0	0.12	4.94	64.3	30.0	0
12	2.72	0	0	0.68	1.66	0.09	0.04	1.48	0.82	62.0	30.1	0.03
16	2.11	0	0.06	0.37	1.71	0	0.04	0.38	0.12	65.3	29.8	0
20	3.51	0	0	1.16	4.33	0.48	0.11	4.35	3.63	49.4	32.0	0.08

2.3.6.3 MICROSCOPIC IMAGES OF CORRODED MILD STEEL COUPONS IN THE SEAWATER/SEDIMENT SYSTEM USING THE STEREO MICROSCOPE

After 20 weeks of incubation in the stimulated seawater/sediment system, the biofilm from the coupons surfaces was removed and the mild steel coupon was then analyzed using the stereo microscope. Microscopic images of these biofilm-free mild steel coupons are shown in Figure 2.11. The microscopic images obtained indicate that during the experiment, minimal corrosion products and deposit formation were observed in the autoclaved control (Figure 2.11a). A distinct uneven surface morphology can be noted on the coupon surface in the non-autoclaved sample (Figure 2.11b) as compared to the autoclaved control. In the sample supplemented with nitrates, pitting and the formation of products as a result of the corrosion process are prevalent (arrows in Figures 2.11c-d). The surface layers appear to be fissured and uneven in the nitrate treated systems indicating severe corrosion, and an abundance of corrosion products were observed on the surface of the metal. A reddish-brown deposit was observed covering the surface of the metal in the non-autoclaved sample (Figure 2.11b) and the sample with additional sodium nitrate (Figure 2.11c), which is characteristic of the main components, iron and oxygen (Table 2.4), present in the formation of corrosion products.

Mild steel coupons exposed to the stimulated seawater/sediment system for 10 months were visualized under the stereo microscope without removal of biofilm or corrosion products as shown in Figure 2.12. An extensive growth of biofilm or corrosion products was evident on the surface of the coupon in the non-autoclaved sample (Figure 2.12b) as compared to the autoclaved control (Figure 2.12a). The black deposit found on the surface of the coupon in the sample with additional sodium nitrate (Figure 2.12c) beneath the reddish-film, is presumably caused by the growth of SRB. The presence of extensive pitting on the surface of the coupon is evident in the sample supplemented with ammonium nitrate (arrow in Figure 2.12d) and may be the product of metal deterioration by the action and metabolism of corrosion-causing bacteria.

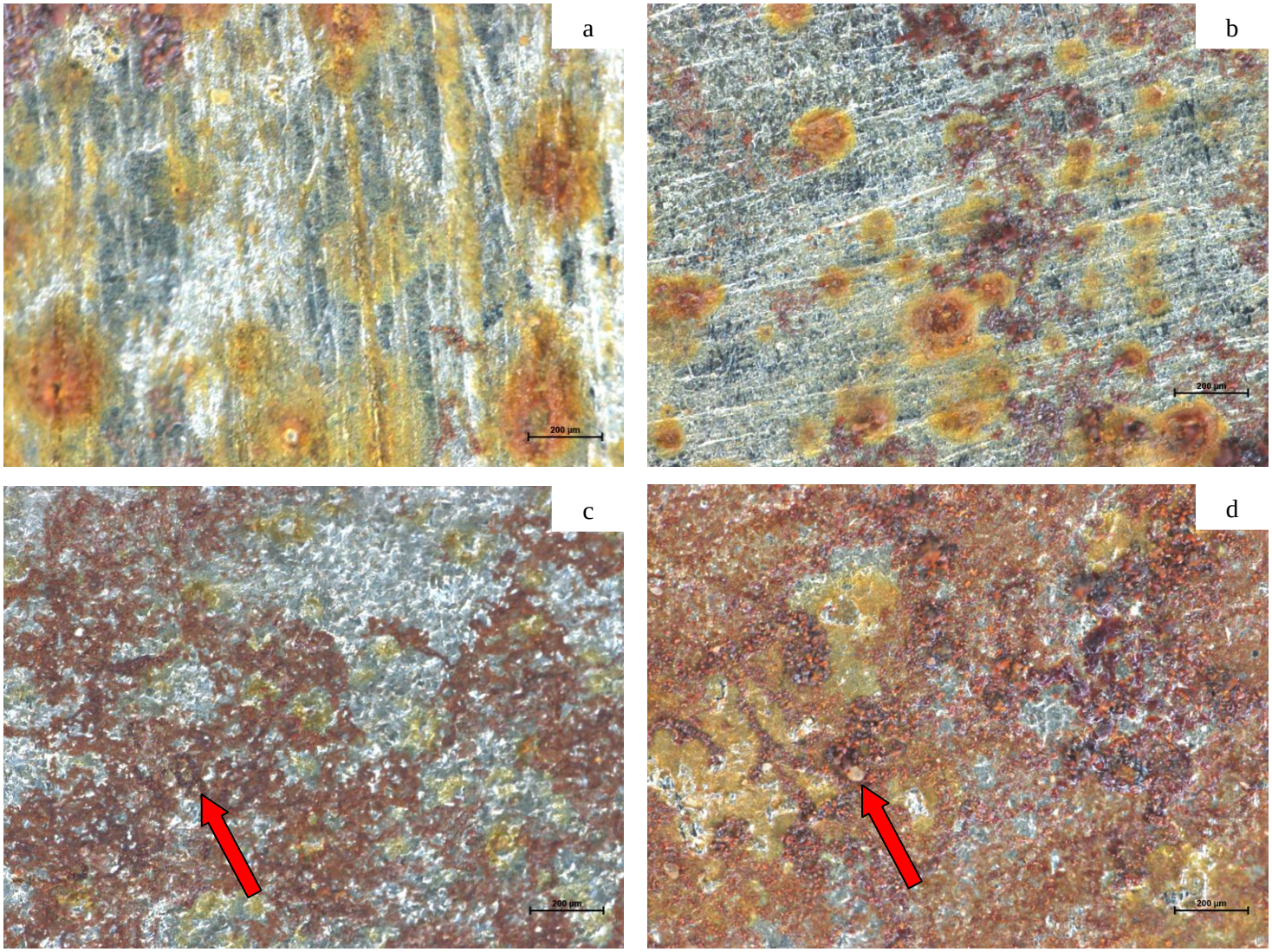

Figure 2.11: Stereo microscopic images of mild steel in seawater/sediment system after 20 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

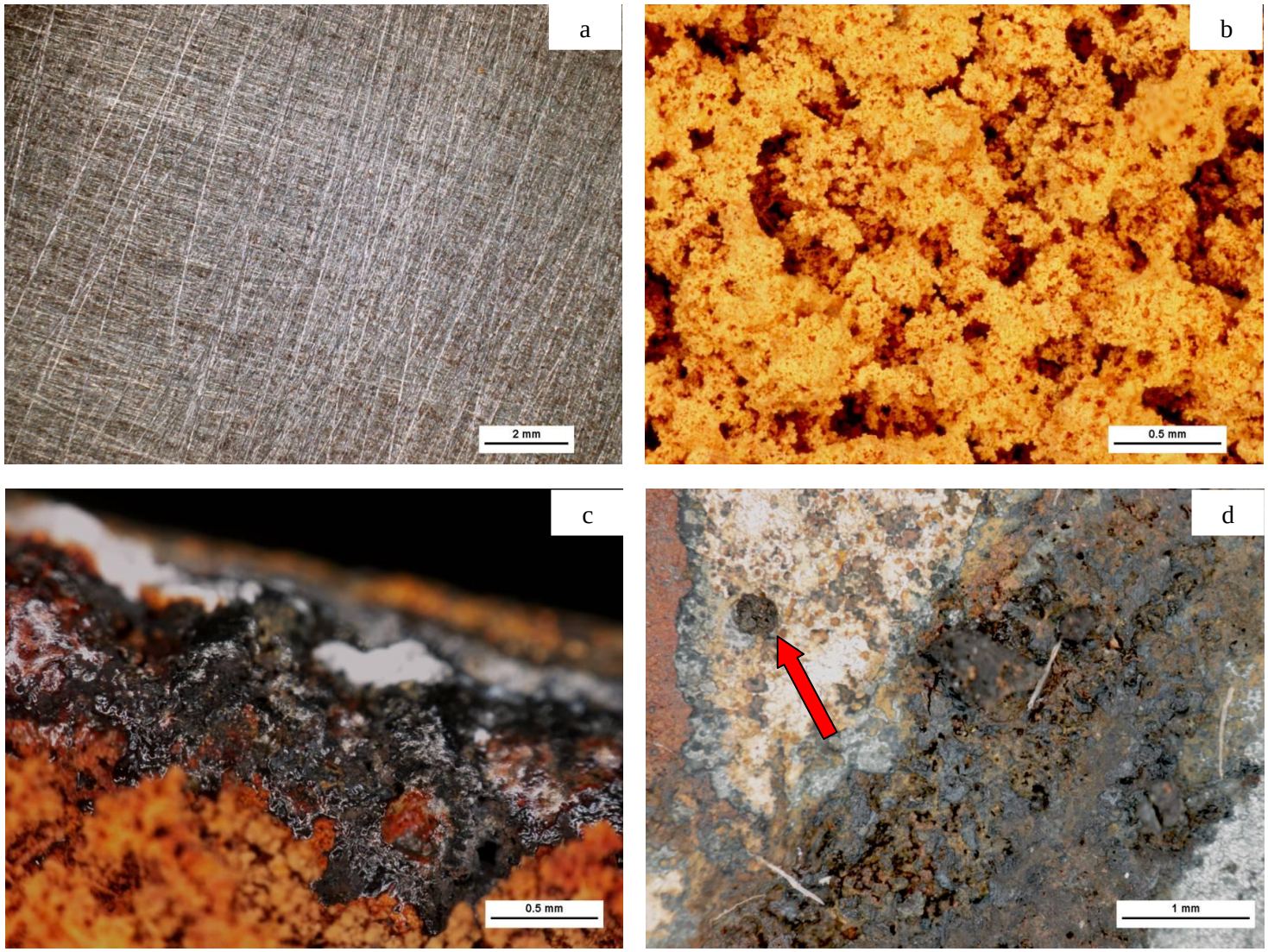

Figure 2.12: Microscopic images of mild steel in seawater/sediment system after 10 months of exposure without removal of biofilm. (a) autoclaved control, (b) presence of corrosion products (c) presence of black deposits, and (d) formation of pits

2.4 DISCUSSION

In this study metal steel coupons were submerged in stimulated seawater/sediment systems and the impact and effectiveness of additional nitrate sources on metal corrosion were investigated. In the nitrate-treated systems (Figure 2.2) the weight loss measurement of the mild steel coupons increased with time. A similar trend was observed in all conditions. This observation can further be supported by the SEM images obtained in this study, depicting corrosion product build up and microbial adherence. Following this, the corrosion rate tends to decrease with further exposure. These later periods have been interpreted as involving corrosion processes that are partly or wholly anoxic, including hydrogen reduction and MIC (Melchers, 2006b). Although this was not examined, the images obtained (Figure 2.12) after 10 months of exposure show rust products and pitting. It is well known that corrosion under anoxic conditions can be severe (Enning *et al*., 2012). It is clear however, that the effect will depend on the time elapsed over which localized anoxic conditions can prevail. This was observed with the mild steel coupons immersed in the stimulated system with additional ammonium nitrate (Figure 2.2).

An increase in weight loss measurements of the coupon were observed with time. On first exposure, a number of processes may occur including pit initiation and for natural seawater, colonization by biofilm and bacteria (Burstein, 2004; Chaves and Melchers, 2011). Figure 2.6d showed visualization of the biofilm formation and microbial adherence on the coupon surface in the system supplemented with ammonium nitrate after four weeks of incubation. This is likely to involve localized anoxic conditions in the surface defects and in the biofilm (Chaves and Melchers, 2011). Previous studies proposed that there were successive changes in the corrosion process, commencing with very rapid pit initiation, followed by an oxygen diffusion rate controlled oxidation process (concentration control, diffusion control) (Melchers, 2006a). As oxygen diffusion conditions take over the overall corrosion process and as rust layers develop, as seen in the stereo microscopic images (Figure 2.11), the relative rate of the anoxic niches will decrease. As the rust products build up however, oxygen diffusion declines and new local anoxic conditions can establish at the corroding surface (Chaves and Melchers, 2011). Eventually, at a period of exposure dependant on water temperature, a relatively sudden increase in the rate of corrosion occurs (Melchers, 2006a).

Tadros and Osman (2012) suggest that the short-term immersion of mild and alloy steels in seawater is proportional to the concentration of dissolved oxygen in the bulk water and the longer-term corrosion is a function of the activity of SRB and is influenced by the concentration of nutrients in the bulk water. From the results obtained, it was observed that the addition of nitrate to this system over a 20 week period increased the weight loss measurements of both the stainless steel and mild steel coupons compared to the autoclaved and non-autoclaved controls (Figures 2.1 and 2.2). This could be a result of the metabolic activities of the corrosion-causing microorganisms being promoted by the additional nitrates, thus increasing the weight loss of the mild steel coupons.

The main cause of metal corrosion is due to the development of microbial biofilms, as detected by SEM, on the metal surface as a result of metabolic activities, especially by SRB that were detected in low counts by MPN (Momba *et al*., 2000; Gu, 2003). It has been proposed that the addition of nitrates into an ecological system can stimulate the growth and metabolic activities of the NRB population (Eckford and Fedorak, 2002). When this particular bacterial population flourishes, it might reduce the activity of the SRB population and thus ultimately reduce the rate of corrosion on steel (Videla and Herrera, 2005; Garces *et al*., 2011). However, an adequate supply of nitrate and sulphate are essential for replication of microorganisms since nitrogen, sulphur and carbon are essential elements for cellular metabolism in microbial life (Puyate and Rim-Rukeh, 2008). For seawater corrosion under anaerobic conditions, SRB and other bacterial metabolism requires the availability of Fe^{+} ions, organic carbon and may involve nitrogen as well (Melchers, 2007; Maluckov, 2012). If these materials are lacking or unfavourable environmental conditions are present, the rate of SRB metabolism will be inhibited. These factors will affect the rate of metal corrosion (Melchers, 2007).

Aramide (2009), showed that the corrosion rate increased with time of exposure to the corrosive medium and that the optimum percentage composition of sodium nitrite required in seawater for optimum corrosion inhibiting performance of mild steel, was determined to be 4 %. In this study the optimum concentration of sodium nitrate and ammonium nitrate was not determined to test the effective corrosion inhibition on the metal coupons. Therefore, it may be that the concentration of the nitrates was too low to stimulate optimum corrosion inhibition. In another study conducted by Mogawer and Brown (2011), it was noted that if nitrite (e.g. calcium nitrite) were added in small quantities, it will act as if there is no inhibitor added to the solution and will therefore have a negative effect on the surface of the alloy. However, the chlorine ions present in seawater will still

attack the surface. In addition, it was found that with an abundant amount of the inhibitor in the seawater, the inhibitor actually acts as a corrosive material and accelerates corrosion (Mogawer and Brown, 2011). These phenomena could be the probable factor contributing to the increase in weight loss measurements seen in the nitrate-treated systems in the current study.

It can be observed that with increasing weight loss there seems to be a decrease in the population growth of both the total heterotrophic and SRB present on the mild steel surface (Figure 2.3 and Figure 2.4). However, SEM observations confirmed the formation of biofilms on the surface of mild steel. Although reduced population growth was observed in the samples with additional nitrates, this could be due to technical difficulties with the MPN technique. From the micrographs obtained, it can be confirmed that there was bacterial colonization and that the microorganisms present in the biofilms were diverse. A disadvantage of the MPN technique is that it requires cultivation and cannot mirror *in situ* conditions and the particular needs of the different physiological groups of bacteria (e.g. temperature, nutrients and syntrophic interactions) (Gittel *et al*., 2009). In a study by Larsen *et al*. (2005), the MPN technique showed at least 100 times lower counts than that obtained by Flourescent *In Situ* Hybridization. The culture independent techniques also demonstrated that SRB made up 10 % of all bacteria present despite being undetectable by the MPN method (Larsen *et al*., 2005; Gittel *et al*., 2009). The web-like structure observed with SEM on the nitrate treated coupon surface indicates the presence of EPS that acts as a binding material for the adhesion of bacterial cells to the biofilm matrix, which is consistent with previous research that EPS is important in the formation of a microconsortium by holding the discrete cells and metal sulfide particles on the metal surface. The water channels in the EPS allow the distribution and circulation of nutrients as well as the bacterial metabolic products within the biofilm (Davey and O'toole, 2000). In the water channels, the microorganisms create a gradient of pH and hydrogen concentration which induces localized corrosion on the steel surface (Jack, 2002). The abundance of lower bacterial species can be explained by interspecies competition or by the direct inhibition by nitrite formed during nitrate reduction (Gittel *et al*., 2009).

A decrease in protein concentration (Table 2.2) on the mild steel coupons was observed in the samples supplemented with nitrates. However, in the nitrate-treated samples an increase in total carbohydrate concentration (Table 2.3) was observed. D'Souza *et al*. (2005) reported higher concentrations of carbohydrates present in biofilms during the first few days of exposure. However, Compere *et al*. (2001) suggested that proteins were the first to adsorb on the surfaces, followed by

carbohydrates, indicating their significance in biofilm development. A decrease in the amount of carbohydrate and protein when the bacteria are in the most active stage, indicates that nutrients are limiting in the system and that bacteria are consuming these substances by degrading the EPS produced by them (Singh *et al*., 2011). Thereafter, the cultivable bacterial concentration starts decreasing, as seen by the MPN counts, (Figures 2.3 and 2.4) while the amount of carbohydrate and protein slightly increases. This may be attributed to the death of bacterial cells which in turn produces more EPS, indicating that bacteria are degrading their own EPS (Lata *et al*., 2012).

The elemental composition of the corrosion deposits can provide information as to the cause of corrosion (Little *et al*., 2006). Energy-dispersive X-ray analysis coupled with SEM has been used in this study to determine the elemental composition of corrosion deposits. EDX was used to assess the elemental abundance at a high spatial resolution over a sampling depth of approximately 1 µm (Lopes *et al*., 2006; Moradi *et al*., 2011). EDX analysis of the mild steel coupons revealed that the main components of the corrosion products were iron and oxygen, as seen by the presence of the reddish-brown deposits observed in the stereo microscopic images (Figure 2.12). This could be indicative of the presence and metabolism of iron-oxidizing bacteria. It is understood that aerobic corrosion is associated with ferric oxides formed as hardened reddish/orange tubercles, although similar oxides may be formed during anaerobic corrosion when products are oxidized during examination (Sherar *et al*., 2011; Zhu *et al*., 2011). There were also trace quantities of calcium, silicon and manganese indicating the presence of iron-oxidizing bacteria that may significantly participate in corrosion deposit formation (Satheesh and Wesley, 2010). An additional contributing factor to the acceleration of the corrosion process is the presence of a weak acidic condition that may facilitate the growth and metabolism of iron-oxidizing bacteria. Table 2.1 indicates a pH of 5.71 for seawater resulting in it being a weak acidic environment. The low pH could be attributed to the acid produced by the iron-oxidizing bacteria and hence accelerate corrosion (Manga *et al*., 2012).

Increased concentrations of calcium, carbon and oxygen were detected in the non-autoclaved sample (Table 2.4) by EDX analyses and thus indicate the formation of calcium carbonate precipitates on the metal surface (Moller *et al*., 2006). In this study high weight percentage values of calcium- (3.023 wt. %) and magnesium-containing (14.05 wt. %) precipitates were detected in the non-autoclaved sample at week 16 (Table 2.4) and a decrease in weight loss measurement (10.685 mg/g) as shown in Figure 2.2 was observed as compared to the nitrate-treated samples in

the stimulated systems. Although SRB could not be isolated in this study and the enumeration of SRB by the MPN technique was low, EDX analysis did detect the presence of sulphur in the corrosion products with higher weight percentage values in the non-autoclaved sample. In addition, increased concentrations of carbon and oxygen detected in all the samples in the stimulated seawater/sediment system indicate the presence of organic material such as bacteria and exopolymeric substances. The observation of calcium and magnesium on the coupon surface in all samples indicates that these compounds play a role in biofilm matrix after microbial settlement (Kokare *et al*., 2009). The unique property of the calcium ion is to promote both specific and non-specific interactions with protein and polysaccharide adhesin molecules at the cell surface (Satheesh and Wesley, 2010). According to literature, the deposition of calcium carbonate and magnesium hydroxide should be able to cause a decrease in the corrosion rate (Moller *et al*., 2006).

It is known that organic compounds are degraded by the presence of heterotrophic bacteria and Fe^{2+} is oxidized to Fe^{3+} by bacterial autotrophs (Khan *et al*., 2012). Manganese oxidizers are able to accumulate and convert the manganese to manganese oxides (Rajasekar *et al*., 2010). Naphta-degrading bacteria converts ferric complex to ferric oxides by the inclusion of oxygen from the degraded products and forms organic complexes (Rajasekar *et al*., 2005; Rajasekar *et al*., 2010). Since ferric/manganese has high affinity for oxygen, it removes oxygen from the degraded product and enhances the formation of ferric/manganese oxides and accelerates corrosion (Maruthamuthu *et al*., 2005; Rajasekar *et al*., 2010). This is observed in the sample stimulated with sodium nitrate with an increase in weight loss measurements observed at week 20 (Figure 2.2) and an increase in weight percentage value for both iron and manganese (Table 2.4). All living organisms contain ATP; therefore, a phosphorous peak in an EDX spectrum can be related to cells associated with the corrosion products (Little *et al*., 2006). Increased weight percentage of phosphorous (Table 2.4) was detected in the sample supplemented with ammonium nitrate with increasing weight loss measurements (Figure 2.2) and total heterotrophic bacterial counts were observed (Figure 2.3). The metabolic activities of SRB and manganese-oxidizing bacteria produce surface-bound sulphur and manganese, respectively. The presence of chloride is generally found within crevices and pits found on the metal surface (Little *et al*., 2006).

The increasing use of the biocompetitive exclusion strategy in industries to inhibit SRB activity is a new technology that has successfully demonstrated its potential in corrosion treatment (Videla and Herrera, 2007; Tabari *et al*., 2011). The addition of nutrients that stimulate the growth of competing

NRB would effectively displace SRB from the microbial community, thus inducing a population shift from SRB domination to NRB domination (Videla and Herrera, 2007). Nitrate addition proves to be more efficacious than biocides, is cost-effective and environmentally friendly (Bodkter *et al*., 2008; Halim *et al*., 2012). The addition of nitrates as a substitute for biocides and corrosion mitigation has been widely used with successful field and laboratory trials (Dunsmore *et al*., 2006; Rempel *et al*., 2006; Bodkter *et al*., 2008). However, the impact of nitrate supplementation and the long-term consequences for MIC are poorly understood (Halim *et al*., 2012).

In recent decades, more attention has been given to the phenomena, mechanisms, monitoring and control methods of MIC (Xu *et al*., 2012). A multifaceted alternate approach for the control of biocorrosion is the use of nitrate. However, nitrate supplementation is still a new, albeit promising technology (Kebbouche-Gana and Gana, 2012). In this study the addition of sodium nitrate and ammonium nitrate (5 mM) did not effectively reduce the weight loss measurements of both stainless steel and mild steel coupons, as compared to the autoclaved controls and non-autoclaved samples (Figure 2.1 and Figure 2.2) over a 20 week study period. The decrease in bacterial population observed could be due to environmental conditions becoming adverse such as nutrient limitations, pH, etc. (Hernandez-Gayoso *et al*., 2004). It is likely that nitrate addition operates to various degrees in different environments, but details of the interactions between nitrate-utilizing bacteria, SRB and environmental conditions are not well understood (Sanders, 2003).

The current study depicted that a concentration of 5 mM of sodium nitrate and ammonium nitrate did not significantly reduce the weight loss of mild and stainless steel coupons. The concentration of the nitrates could be too low in order to stimulate the nitrate-reducing bacterial population and hence, inhibit corrosion. Further studies needs to be conducted in order to optimize the concentration of nitrates for corrosion inhibition. It is also recommended that the environment in which the corrosion takes place be evaluated. The environmental condition plays a major role in the corrosion process as is the type of metal being corroded.

In this study, it was shown that even when the bacterial population was stable (Figure 2.3), the corrosion rate continued to increase (Figure 2.2). All previous successful studies utilized seawater from deep ocean environments, where the microbial population and environmental conditions might be significantly different to that examined in the current study. The composition of seawater is nutrient-rich harboring diverse microbial populations. This well-adapted indigenous microflora

could prove to utilize the nitrates optimally thus inhibiting corrosion of metals. However, in the current study, seawater was retrieved from the shoreline and this could be a contributing factor either to the source being limited in nutrients or not contain a vast biological system. Therefore further studies need to be conducted to test the nutrient composition and microbial populations of the natural seawater collected before optimization of the nitrate concentration.

CHAPTER THREE
THE IMPACT OF ADDITIONAL NITROGEN SOURCES IN METAL CORROSION IN A STIMULATED LOAM SOIL SYSTEM

3.1 INTRODUCTION

Soils constitute the most complex environment known to metal corrosion. Corrosion in soils can vary from relatively rapid material loss to negligible effects, depending on the soil environment. The study of soils as a corrosive environment is necessary due to the large number of buried pipelines and tanks, and their deterioration can appear to be a real economical and environmental problem. Soil engineering properties and soil contents are important parameters that influence the soil corrosivity and level of corrosion dynamic (Norhazilan *et al*., 2012). Different parameters such as geological factors (soil type, pH and electrical resistivity), hydrological factors (water flow through ground layers), soil chemical content, microbiological activity or pollution may be more dominant in influencing the dynamics of soil corrosion (Bano and Qazi, 2012; Norhazilan *et al*., 2012). Moisture content in soil will probably have the most profound effect when considering corrosive potential than any other factors. Clay content may also contribute to the dynamic of underground corrosion. This has to do with its characteristics as the finest particle in soil (Norhazilan *et al*., 2012).

The corrosion behavior of structural steels in soil can be divided into two categories: corrosion in disturbed soil and corrosion in undisturbed soil (Bragg, 2008). Buried steel pipelines and tanks in the soil suffer from corrosion (Cunat, 2001). The corrosion rate of steel in disturbed soil is influenced by soil resistivity, pH, chloride content, sulphate content, sulphide ion content, soil moisture and oxygen content within the soil. In undisturbed soils, the oxygen at levels a few feet below the ground line, or in and below the water table zone, are extremely deficient. Steel pilings are not appreciably affected by corrosion, regardless of the soil types or soil properties (Bragg, 2008).

Although mild and stainless steels are the most frequently used engineering materials in the oil and gas industries, the material used for most equipment is mild steel due to costs (Starosvesky *et al.*, 2000; Oladele and Okoro, 2011). However, mild steel corrodes easily because all common

structural metals form surface oxide films when exposed to pure air (Oladele and Okoro, 2011). The main requirement for stainless steels is that they should be corrosion resistant for a specified application or environment (Atta *et al*., 2011). However, stainless steels are known to suffer from localized corrosion by the presence of microorganisms (Starosvesky *et al.,* 2000).

Microorganisms can change the electrochemical conditions at the metal–solution interface, leading to either the induction/acceleration of corrosion or corrosion inhibition (Videla and Herrera, 2005). Considerable scientific attention has been devoted to investigating the microbial corrosion process of steel and iron in the presence of SRB (Starosvesky *et al.,* 2000). SRB are non-fermentative anaerobes that obtain their energy for growth from the oxidation of organic substances using inorganic sulfur oxy-acids or nitrate as terminal electron acceptors whereby sulfate is reduced to sulfide (Lee and Characklis, 1993; Feio *et al.,* 2000). However, the mechanism of how SRB influence the anaerobic corrosion of ferrous metal continues to be controversial (Rainha and Fonseca, 1997). The sulphate concentration in a system in most cases has a direct influence on the growth and activity of SRB and the amount of sulphide produced (Sanders, 1988). Fonseca *et al*. (1998) tested the corrosion of mild steel under different media both with and without sulphate ions and found that the initiation of biocorrosion due to SRB only occurred in the presence of sulphate species. Mohanty *et al*. (2000) also found that a high sulphate concentration in the medium could inhibit the sulphate reduction rate of SRB.

Microorganisms can contribute to corrosion inhibition by different mechanisms: neutralizing the action of corrosive substances present in the environment; forming protective films or stabilizing a pre-existing protective film on a metal; and inducing a decrease in the medium corrosiveness. Microbial inhibition and microbial induction of corrosion are rarely linked to a single mechanism of a single species of microorganisms. Either the corrosive or the inhibitory action of bacteria develops on biofilm on metal surfaces where complex biofilm/protective films occur. The main mechanisms of bacterial corrosion inhibition are always linked to a marked modification of the environmental conditions at the metal–solution interface due to biological activity (Videla and Herrera, 2005).

The use of biocompetitive exclusion (BE) strategies is increasing in attempts to inhibit MIC. This strategy involves the addition of nutrients that stimulate the growth of competing bacterial populations (namely NRB) to effectively displace SRB from a microbial community. Thus, the addition of nitrate can induce a shift in the dominant population from SRB to NRB. The use of

nitrate to control SRB and H_2S production in oil fields is nowadays a proven biotechnology whose effectiveness has been shown both in the laboratory and in several field studies (Videla and Herrera, 2005). Nitrites have also been previously reported to be an effective corrosion inhibitor. Its popularity as a corrosion inhibitor arises from its relative low costs (Garces *et al*., 2011). The replacement of generally toxic biocides using an environmentally friendly approach has proven to be successful, not only to control sulfide levels but also to reduce MIC effects (Videla and Herrera, 2005). However, the supplementation of nitrate source failed to slow down the corrosion process in the seawater/sediment system as demonstrated in the previous chapter. Therefore, this chapter describes the impact and effectiveness of sodium nitrate and ammonium nitrate, on metal coupons in a simulated loam soil system. The steel samples were analyzed to determine whether the nitrates will cause a differential effect on the weight measurements in a different environmental set-up.

3.2 MATERIALS AND METHODS

3.2.1 SAMPLE COLLECTION AND PREPARATION

Loam soil was collected in Shallcross (KwaZulu-Natal), air-dried and homogenized. The soil was then passed through a 7.5 mm (porous aperture) Madison Test Sieve and stored at 4 °C prior to further analysis. pH and moisture content of the soil was analyzed following standard protocols (McCauley *et al*., 2003). The metal coupons used in this study were obtained from the Academic Instrumentation Unit (University of KwaZulu-Natal, Westville). Dimensions and preparations of the coupons were described in Section 2.2.1 in Chapter 2.

3.2.2 CONSTRUCTION OF THE LOAM SOIL SYSTEM AND DETERMINATION OF CORROSION BY WEIGHT LOSS MEASUREMENTS

The experiments were constructed similarly to the set-up of the seawater/sediment system as described by Orfei *et al*. (2006) and in Section 2.2.2 in Chapter 2. The coupons were removed in regular intervals and were treated with 20 % HCl, scrubbed, cleaned and dried and the weight loss of the coupons was determined as described in Section 2.2.2 in Chapter 2.

3.2.3 ENUMERATION OF THE TOTAL HETEROTROPHIC AND SULPHATE-REDUCING BACTERIAL POPULATION PRESENT ON THE SURFACE OF THE COUPONS

Sampling for microbiological analyses was performed by swabbing duplicate coupons with cotton swabs (25 mm Gamma sterilized) over a period of time. Enumeration and quantification of the total heterotrophic and sulphate-reducing bacterial population was determined using the Most Probable Number technique as described in Section 2.2.3 in Chapter 2.

3.2.4 ANALYSIS OF THE CARBOHYDRATE CONTENTS OF THE BIOFILM DEVELOPED ON THE COUPON SURFACE

The carbohydrate contents of the biofilm developed on the coupon surface were calculated using standard D-glucose curves as described by Beech *et al*. (2000). The samples were analyzed using the DNS assay for total reducing sugars according to the methodology described in Section 2.2.4 in Chapter 2.

3.2.5 ESTIMATION OF THE PROTEIN CONTENTS OF THE BIOFILM DEVELOPED ON THE COUPON SURFACE

Protein measurements were performed using the Bradford assay as described by Beech *et al*., (2000). The sample concentration was calculated using a standard bovine serum albumin curve as described in Section 2.2.5 in Chapter 2.

3.2.6 MICROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS

Mild steel coupons were subjected to chemical fixation according to a modified method of Faimali *et al*. (2004). The coupons were examined using the SEM Leo 1450 and the Jeol JSM 6100 SEM equipped with a Bruker EDX detector as described in Section 2.2.6 in Chapter 2.

3.2.7 STATISTICAL ANALYSIS

Data were analyzed using SPSS for windows (version PASW18, SPSS Inc., Chicago, IL). A one-way ANOVA was used to test mean differences in weight losses of the metal coupons over time. A p-value of <0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 PHYSICAL CHARACTERIZATION OF THE SAMPLING SITE

Table 3.1 shows the physical characteristics of loam soil. Loam soil used in this study was more alkaline (pH 8.09) and had a lower percentage of water content (3.91 %) as compared to 8.04 % as obtained in the seawater/sediment system (Chapter 2).

Table 3.1: The physical characteristics of the sampling site

	LOAM SOIL
pH	8.09
PERCENTAGE WATER CONTENT	3.91 %
PERCENTAGE DRY MASS	96.09 %

3.3.2 WEIGHT LOSS MEASUREMENTS OF STAINLESS STEEL COUPONS FROM THE LOAM SOIL SYSTEM

The results of the weight loss measurements of stainless steel coupons immersed in the stimulated loam soil system are shown in Figure 3.1. The stainless steel coupons demonstrated a significant lower weight loss in the loam soil system (mg/g of stainless steel coupon) compared to those in the seawater/sediment system after 20 weeks as shown in Figure 2.1. The corrosion levels of stainless steel coupons in autoclaved and non-autoclaved controls increased slowly throughout a 20 week study period from 0.08 mg/g at week 2 to 0.13 mg/g at week 20, respectively except at week 8 (Figure 3.1). A significant increase in weight loss of all stainless steel 304 coupons (Figure 3.1) was observed in all loam samples at eight weeks as compared to that observed at week 2 and week 4. The stainless steel coupons in all nitrate treated systems had similar weight loss levels with both autoclaved and non-autoclaved controls until week 12 and demonstrated a lower weight loss at week 16 and week 20. The differences between nitrate treated samples and the controls are significant ($p < 0.05$). Due to the slow corrosive nature of the stainless steel coupons and no significant changes under SEM in this study, the stainless steel coupons were excluded in the remaining study.

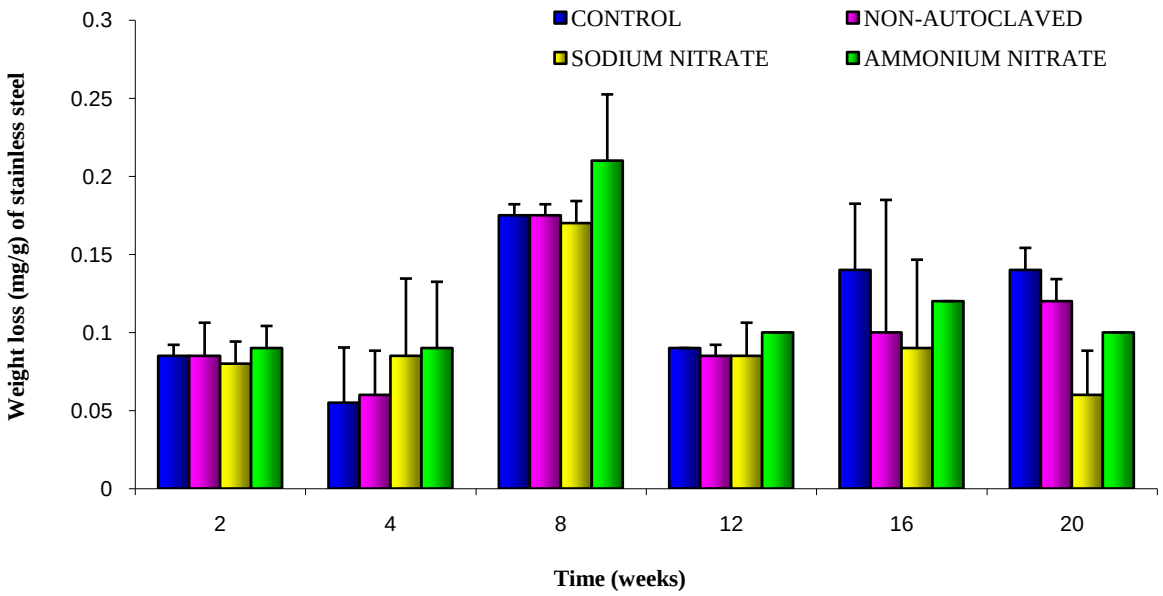

Figure 3.1: Weight loss of stainless steel coupons in loam soil for a period of 20 weeks

3.3.3 WEIGHT LOSS MEASUREMENTS OF MILD STEEL COUPONS FROM THE LOAM SOIL SYSTEM

The weight loss measurements of mild steel coupons immersed in a stimulated loam soil system are shown in Figure 3.2. The mild steel coupons demonstrated a significant lower weight loss in the nitrate treated systems than that observed in the autoclaved control system. An increase in weight loss of the mild steel coupons was observed in the loam soil samples treated with sodium nitrate throughout the 20 week study period from 2.52 mg/g at week 2 to 26.73 mg/g at week 20, except at week 16. An increase in weight loss of the mild steel coupons was observed in the non-autoclaved control from week 2 (1.66 mg/g) to week 16 (22.83 mg/g). However, at week 20 (2.46 mg/g), lower weight loss of the coupons was observed in the non-autoclaved control sample in the loam soil system.

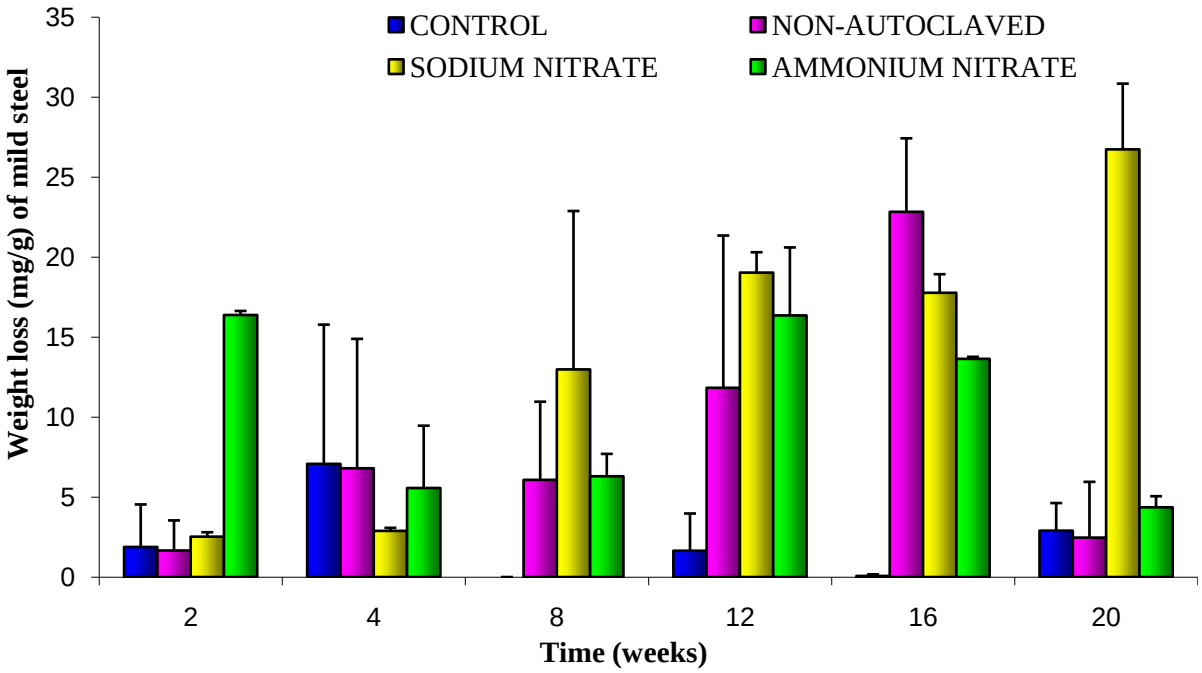

Figure 3.2: Weight loss of mild steel coupons in loam soil over a period of 20 weeks

3.3.4 ENUMERATION OF TOTAL HETEROTROPHIC BACTERIA AND SULPHATE-REDUCING BACTERIAL POPULATIONS ON THE METAL SURFACE

The total heterotrophic and SRB populations extracted from the biofilm samples on the surface of the mild steel coupons were enumerated using the Most Probable Number technique. This was expressed as the MPN/ml based on the results obtained for MPN corrected for bias (Salama) using the Most Probable Number Calculator©.

The results of the enumeration of the total heterotrophic bacterial population extracted from the surface of the mild steel coupons immersed in the stimulated loam soil system are shown in Figure 3.3. The growth of the total population of heterotrophic bacteria in the non-autoclaved control increased from week 2 (107.30 MPN/ml) to week 4 (465.582 MPN/ml), but thereafter decreased throughout the rest of the study (55.115 MPN/ml at week 20). The total heterotrophic bacterial population in the nitrate treated systems showed increased population growth towards the end of the study period compared to the autoclaved and non-autoclaved controls. In the loam soil system stimulated with sodium nitrate, the initial population growth was observed at week 2 (88.13 MPN/ml). However, at week 4 and week 8 (17.251 MPN/ml and 8.077 MPN/ml respectively), the

growth of the population decreased and then increased from week 12 (201.185 MPN/ml) to week 20 (106.695 MPN/ml). The population growth of the total heterotrophic bacteria in the loam soil system stimulated with ammonium nitrate increased slowly throughout the 20 week study period, except for week 12 (37.155 MPN/ml) and week 20 (54.793 MPN/ml). However, there was no significant difference ($p > 0.05$) among the treatments of the total heterotrophic bacterial populations enumerated.

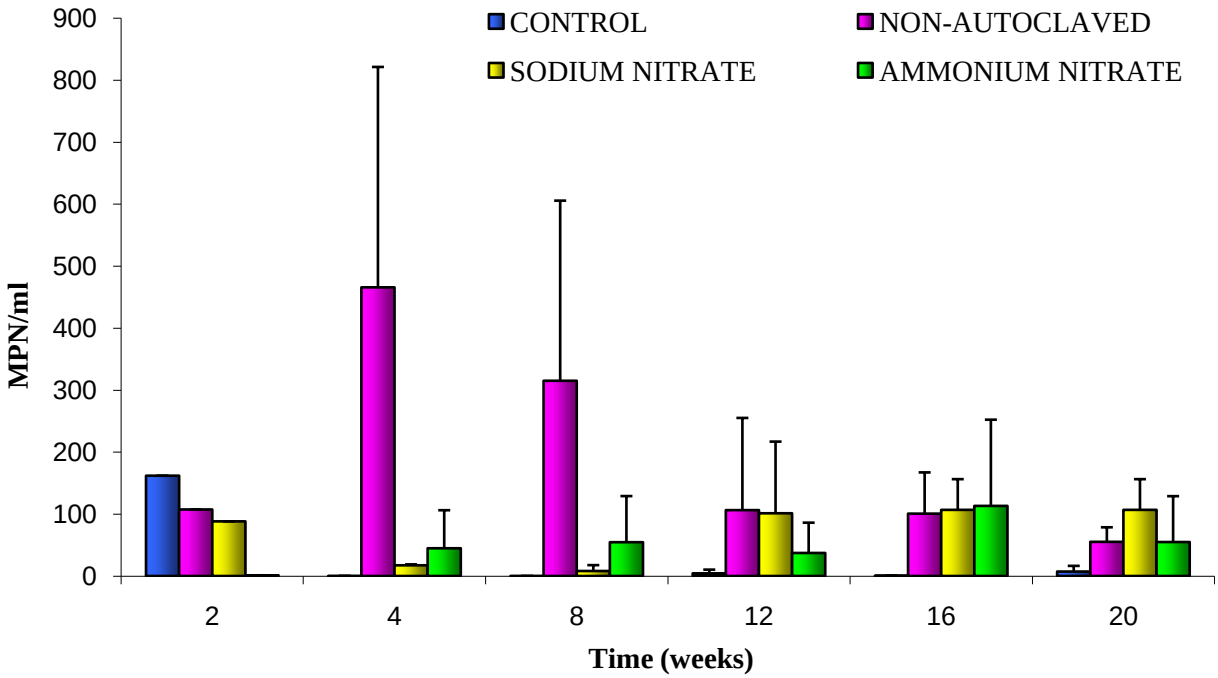

Figure 3.3: Enumeration of total heterotrophic microorganisms on mild steel coupons in loam soil over a period of 20 weeks

The enumeration of the population growth of SRB extracted from the surface of mild steel coupons immersed in the stimulated loam soil system are shown in Figure 3.4. During the initial sampling times of the 20 week study period, a decrease in the population growth of the anaerobic bacteria was observed. An increase in growth of the SRB population was observed in the nitrate treated loam soil systems at week 8 (42.815 MPN/ml and 46.415 MPN/ml for sodium nitrate and ammonium nitrate treated systems respectively) and week 12 (73.284 MPN/ml and 296.745 MPN/ml for sodium nitrate and ammonium nitrate treated systems respectively). A decrease in the population of SRB was observed in all samples in the stimulated loam soil system at week 16 and week 20 however, population growth was not significantly different in any sample ($p>0.05$).

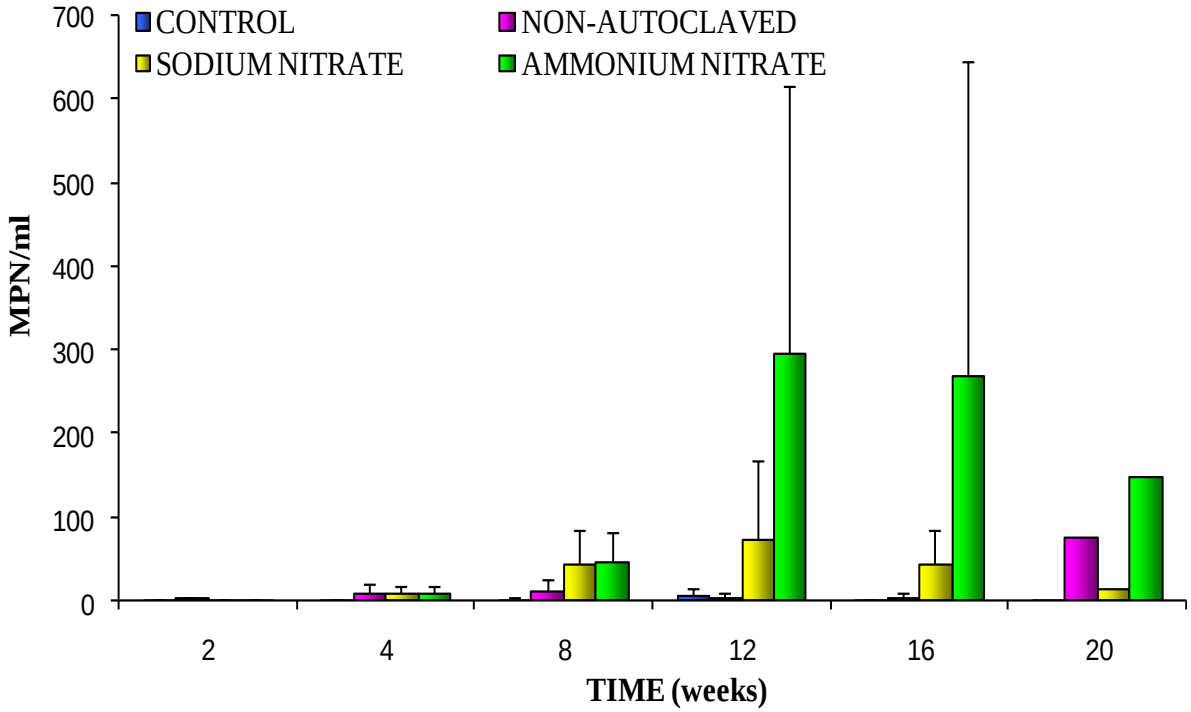

Figure 3.4: Enumeration of sulphate-reducing microorganisms on mild steel coupons in loam soil over a period of 20 weeks

3.3.5 THE EVALUATION OF THE CARBOHYDRATE AND PROTEIN CONCENTRATION PRESENT IN THE BIOFILM SAMPLES ON MILD STEEL SURFACE

The protein concentrations of the biofilm formed on the surface of the mild steel coupons immersed in the stimulated loam soil system are shown in Table 3.2. The biofilm formed on the mild steel coupons demonstrated a significantly greater concentration of proteins in the non-autoclaved control and the nitrate treated systems compared to the autoclaved control in loam soil. The autoclaved control sample showed a minimal concentration of protein at week 4 (0.997 mg/ml) and week 20 (0.044 mg/ml) with no protein concentration determined from week 8 to week 16. An increase in protein concentration was estimated at week 8 (7.164 mg/ml) in the non-autoclaved control. However, the protein concentration lowered over the study period to 4.2311 mg/ml at 20 weeks. In the nitrate treated samples, an increase in protein concentration was determined at week 8 (6.927 mg/ml) for samples stimulated with sodium nitrate and at week 12 (4.779 mg/ml) for samples with ammonium nitrate stimulation. However, the samples treated with ammonium nitrate demonstrated a significantly lower concentration of proteins over time with 2.3526 mg/ml

determined at week 20 as compared to the non-autoclaved sample ($p < 0.05$). The protein concentration decreased at 16 weeks (1.862 mg/ml) in the samples supplemented with sodium nitrate but thereafter, increased at week 20 (4.489 mg/ml).

Table 3.2: Total protein concentration (mg/ml) in biofilm samples extracted from mild steel coupons in loam soil over a period of 20 weeks

INCUBATION TIME	**4**	**8**	**12**	**16**	**20**
Total Protein Concentration (mg/ml)					
CONTROL	0.997	0	0	0	0.044
NON-AUTOCLAVED	0	7.164	5.589	4.289	4.231
SODIUM NITRATE	1.173	6.928	4.218	1.862	4.489
AMMONIUM NITRATE	0	2.608	4.779	1.224	2.353

The carbohydrate concentrations of the biofilm formed on the surface of the mild steel coupons immersed in the stimulated loam soil system are shown in Table 3.3. The determination of the concentration of carbohydrates at week 4 (4.007 mg/ml) in the non-autoclaved samples were significantly greater compared to the autoclaved control ($p < 0.05$). An increase in carbohydrate concentration in the autoclaved control was determined from week 4 (1.491 mg/ml) to week 12 (4.281 mg/ml). However, a decrease in concentration was thereafter determined with 3.993 mg/ml of carbohydrates estimated at week 20. The biofilms formed on the surface of mild steel coupons demonstrated a significantly lower concentration of carbohydrates in the non-autoclaved sample and the nitrate treated systems compared to the autoclaved control in loam soil. In the sample treated with ammonium nitrate, carbohydrate concentration was greater at week 4 (4.191 mg/ml) but thereafter decreased with 1.096 mg/ml of carbohydrate determined at week 20. Lower concentration of carbohydrate was determined at week 4 for the sample supplemented with sodium nitrate (2.157 mg/ml) as compared to the ammonium nitrate treated system. No carbohydrates were determined at week 8 and week 20 for the sample treated with sodium nitrate.

Table 3.3: Total carbohydrate concentration (mg/ml) in biofilm samples extracted from mild steel coupons in loam soil over a period of 20 weeks

INCUBATION TIME	**4**	**8**	**12**	**16**	**20**
	Total Carbohydrate Concentration (mg/ml)				
CONTROL	1.491	3.997	4.281	4.254	3.993
NON-AUTOCLAVED	4.007	0	0	0	0
SODIUM NITRATE	2.157	0	0.033	2.893	0
AMMONIUM NITRATE	4.191	0.779	1.468	2.618	1.096

3.3.6 SCANNING ELECTRON MICROSCOPY AND ELECTRON DIFFRACTION X-RAY ANALYSES OF CORRODED MILD STEEL COUPONS IN LOAM SOIL

3.3.6.1 SCANNING ELECTRON MICROSCOPY (SEM)

Observations of the mild steel coupons immersed in the stimulated loam soil system over a 20 week study period were conducted by SEM. Figure 3.5 shows the SEM image of mild steel coupons after 4 weeks of incubation. The autoclaved control (Figure 3.5a) displayed no microbial activity or biofilm formation as compared to the other samples. The non-autoclaved sample revealed microbial growth, as indicated by the presence of cocci-shaped bacteria, and adherence to the metal surface (arrow in Figure 3.5b) as compared to the autoclaved control. In the nitrate treated systems, abundant biological formation, indicated by the presence of cocci-shaped bacteria, and activity was present on the surface of the coupon embedded in soil (arrows in Figure 3.5c and d).

Microbial adherence and needle-like corrosion products were observed in the non-autoclaved sample after 12 weeks of incubation (arrow in Figure 3.6b) as compared to the autoclaved control (Figure 3.6a). Substantial biofilm formation was observed in the sample supplemented with sodium nitrate (Figure 3.6c). Abundant microbial adherence was observed in the sample with additional ammonium nitrate (arrow in Figure 3.6d).

After 20 weeks of incubation, SEM confirmed that there was effective bacterial colonization on the metal surface and needle-like corrosion products in the non-autoclaved sample (arrow in Figure 3.7b) as compared to the autoclaved control (Figure 3.7a). A heterogeneous microbial layer with copious amounts of microorganisms was observed on the steel surface in the nitrate treated systems.

A heterogeneous microbial layer with the presence of cluster formation and needle-like corrosion products was observed on the surface of the mild steel coupons in the non-autoclaved sample and the nitrate treated systems over the 20 week study period. However, in the sample supplemented with sodium nitrate, the formation of pockets with the appearance of being burst open were also observed (arrow in Figure 3.7c). In the sample treated with ammonium nitrate a deep biofilm layer was observed with effective bacterial colonization (arrow in Figure 3.7d).

3.3.6.2 ENERGY DISPERSIVE X-RAY (EDX) ANALYSIS OF MILD STEEL COUPONS

Mild steel coupons were extracted from the stimulated loam soil system after 4, 8, 12, 16 and 20 weeks of incubation and analysed for its elemental composition using an SEM equipped with a Bruker EDX detector. The different elemental compositions of the mild steel coupons in the autoclaved and non-autoclaved controls immersed in a stimulated loam soil system over a 20 week study period are shown in Table 3.4. The elemental composition of the untreated mild steel coupon comprised mainly of iron (69.66 wt. %), oxygen (30.08 wt. %) and manganese (0.106 wt. %). In addition, trace amounts of calcium, aluminium and silicon were also detected on the coupon surface (0.016 wt. %, 0.07 wt. % and 0.02 wt. % respectively).

During the initial stages of the experiment (week 4), EDX analysis of the biofilm sample on the mild steel coupons confirmed that the main components in all samples were iron and oxygen as shown in Figure 3.8. Increased concentrations of carbon were detected in the nitrate treated systems, (3.516 wt. % and 3.696 wt. % for sodium nitrate, Figure 3.8c; ammonium nitrate, Figure 3.8d supplemented samples respectively) as compared to the autoclaved and the non-autoclaved controls (2.463 wt. % and 2.4 wt. % respectively).

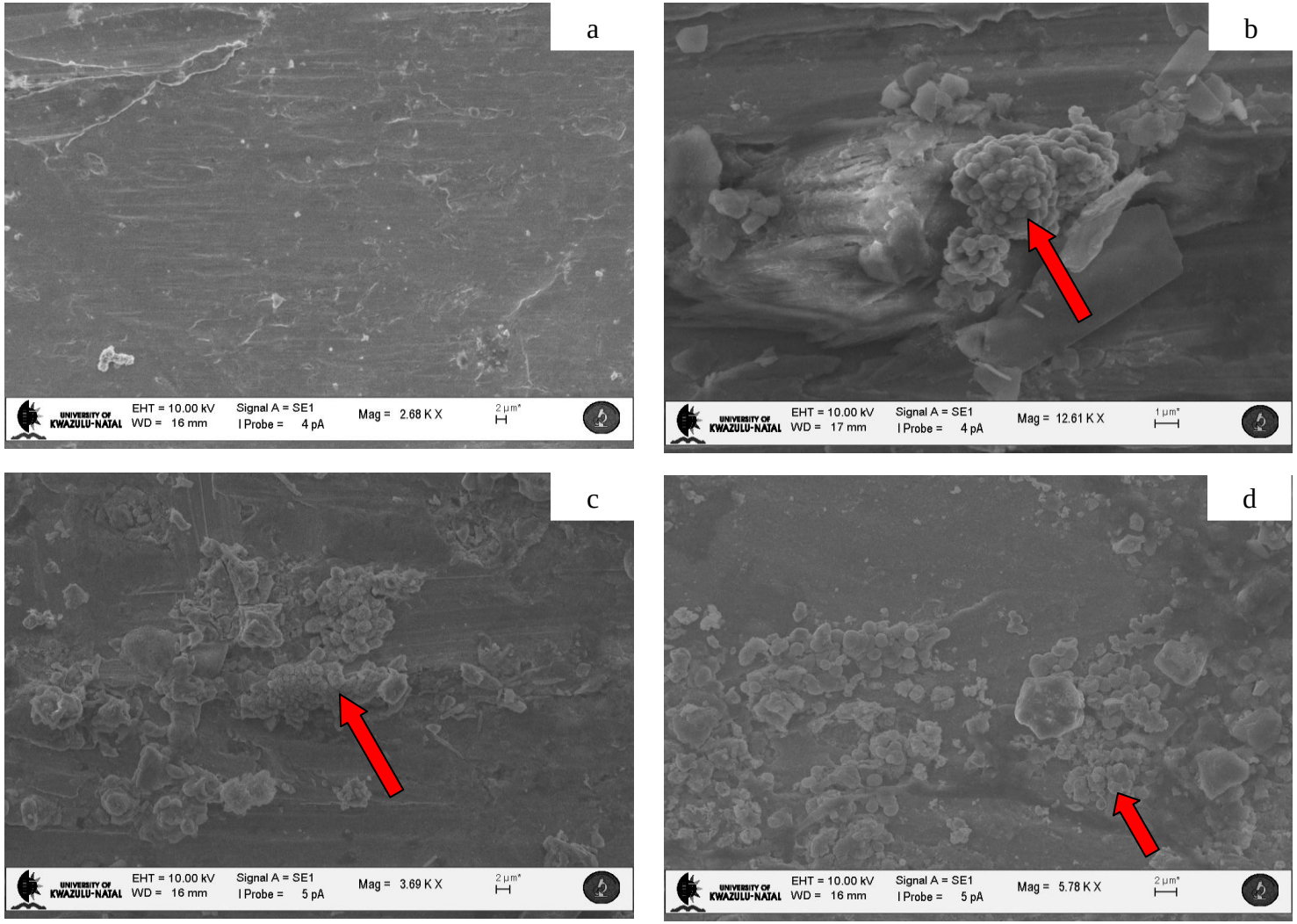

Figure 3.5: Scanning electron image of mild steel in loam soil after 4 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

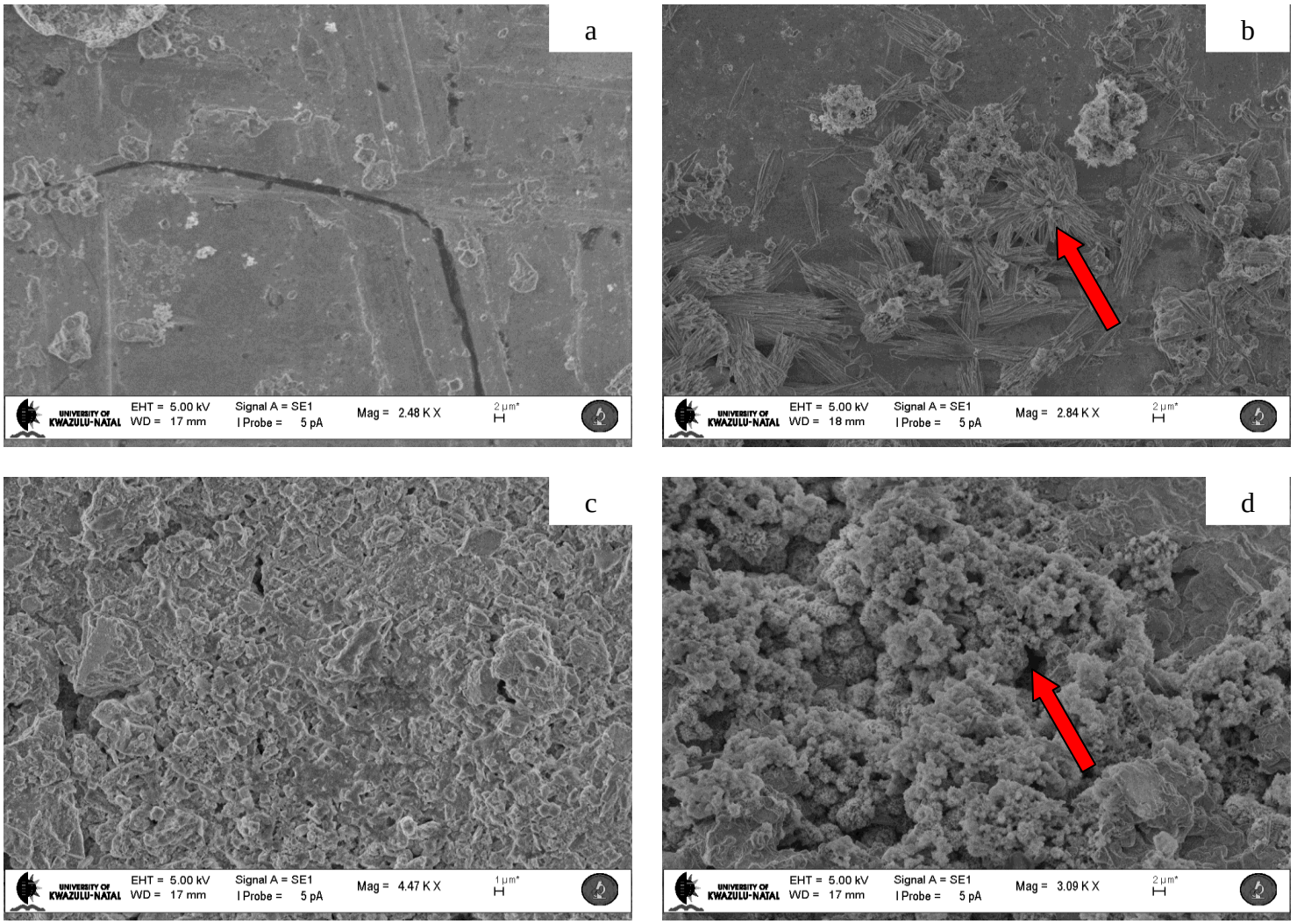

Figure 3.6: Scanning electron image of mild steel in loam soil after 12 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

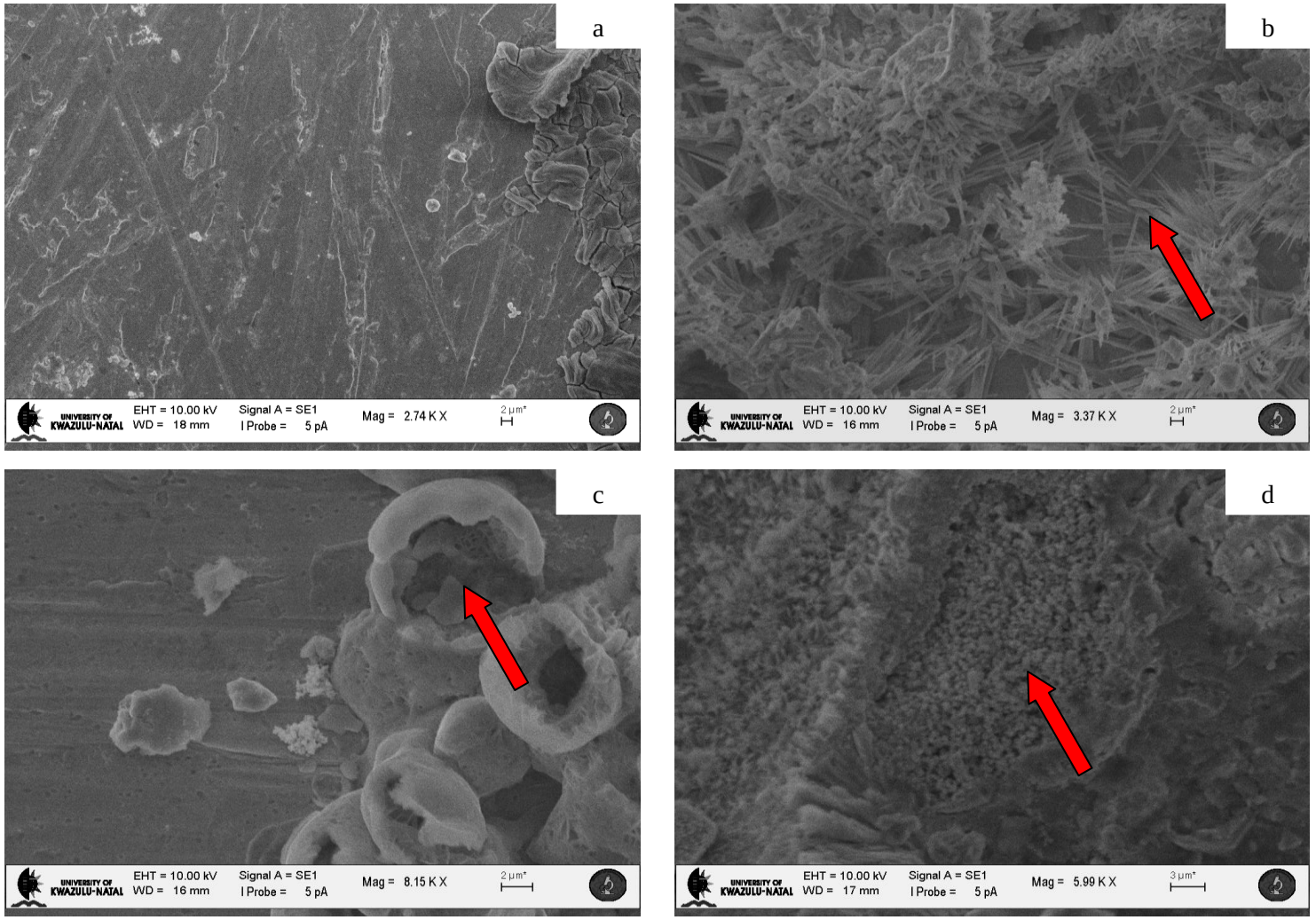

Figure 3.7: Scanning electron image of mild steel in loam soil after 20 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

EDX analysis of the coupons revealed that the main components in all samples are iron and oxygen. There was a high concentration of fluorine (3.376 wt. %) detected at eight weeks but thereafter this concentration decreased. High concentrations of carbon (4.076 wt. %) and calcium (3.796 wt. %) were detected at 16 weeks on the mild steel coupons. Decreased concentration of manganese, magnesium, sodium, aluminium and silicon were detected in the autoclaved control. Low concentrations of sulphur were detected at eight and 20 weeks (0.046 wt. % and 0.02 wt. % respectively) on the mild steel coupons in the non-autoclaved control. Increased concentrations of carbon (4.846 wt. %) and calcium (7.976 wt. %) were observed in this sample at 20 weeks. Increased concentration of silicon (5.113 wt. %) were observed at 20 weeks whereas lower concentrations of magnesium, manganese, calcium and phosphorous were detected in the non-autoclaved control in the loam soil system. Increased concentrations of calcium (33.92 wt. %) and silicon (5.516 wt. %) were detected at eight weeks on mild steel coupons in the system stimulated with sodium nitrate.

A high concentration of carbon (3.873 wt. %) was detected at 12 weeks however, the concentration of calcium (0.366 wt. %) was reduced in this treated sample in loam soil. Increased concentrations of carbon (3.696 wt. %) and calcium (2.033 wt. %) were detected at week 4 and week 20 respectively, in the system stimulated with ammonium nitrate. Trace amounts of manganese, magnesium, aluminium and silicon were detected on mild steel coupons in this treated system. No sulphur was detected in both nitrate treated systems.

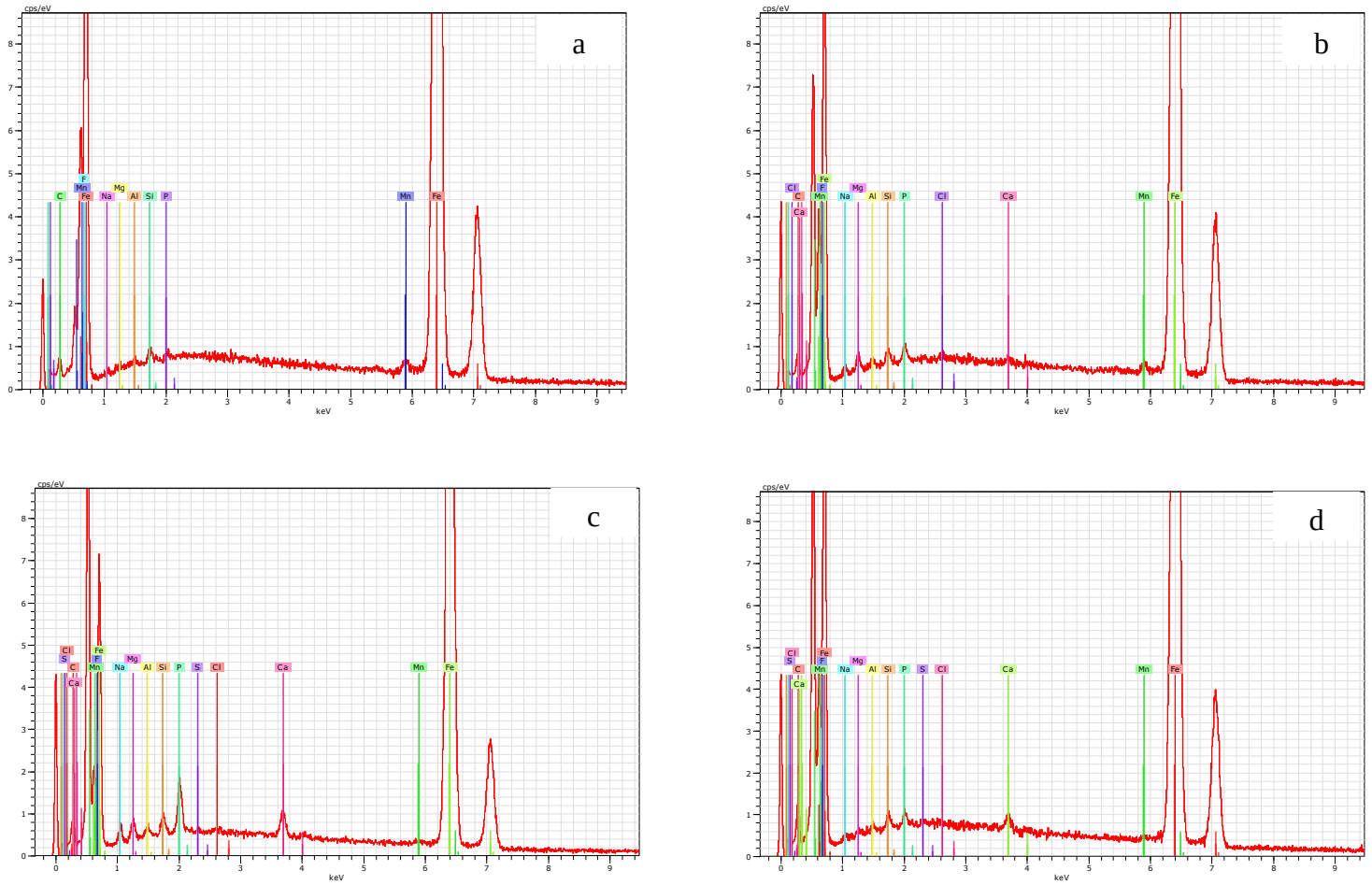

Figure 3.8: EDX spectra showing different elemental composition on the surface of the mild steel coupons after 4 weeks of incubation. (a) Control, (b) non-autoclaved system, (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate

Table 3.4: Percentage values of different elemental compositions found on the mild steel coupons in the stimulated loam soil system using the Energy Dispersive X-Ray Analysis

TIME (WEEK)	C	F	Mn	Na	Mg	Al	Si	P	Ca	Fe	O
AUTOCLAVED CONTROL											
4	2.463	0.796	0.563	0.32	0.17	0.576	2.26	0.076	0.423	61.82	30.23
8	0	3.376	0	0.573	0	0.05	0.163	0.106	0	66.51	29.22
12	3.26	0	0.486	0.72	0.163	0.443	0.343	0.173	0.18	64.97	29.25
16	4.076	0	0.13	0.566	0.63	1.016	1.923	0.113	3.796	57.59	29.98
20	2.846	0	0.2	0.616	0.34	0.283	0.446	0.213	1.1	64.52	29.44
NON-AUTOCLAVED CONTROL											
4	2.4	0.62	0.42	0.416	0.243	0.34	0.663	0.133	0.216	64.89	29.50
8	0	2.55	0	0.886	0.566	0.473	1.033	1.116	0.636	61.95	30.66
12	3.643	0	0.463	0.53	0.25	0.376	0.376	0.273	1.023	63.78	29.24
16	2.543	0	0.34	1.273	0.453	0.35	1.113	0.793	0.933	62.12	30.07
SODIUM NITRATE											
4	3.516	0.233	0.15	0.326	0.213	0.076	0.1	0.363	0.206	65.34	29.00
8	0	0	0	0	0.266	1.07	5.516	0	33.92	26.77	32.45
12	3.873	0	0.52	2.333	0.333	1.513	3.253	1.043	0.366	55.71	30.88
16	2.47	0	0.313	0.54	0.446	0.256	0.513	0.256	0.08	65.41	29.68
20	2.463	0	0	1.623	0.22	0.573	1.82	0.486	0.983	61.33	30.2
AMMONIUM NITRATE											
4	3.696	0.72	0.21	0.24	0.066	0.03	0.106	0.08	0.086	65.98	28.75
8	0	0	0.396	1.15	0.113	0.1	0.203	1.01	0.216	66.35	30.43
12	3.066	0	0	0.453	0	0.233	0.333	0	0.186	42.93	19.10
16	3.11	0.616	0.233	0.933	0.163	0.346	1.11	0.38	0.193	63.2	29.65
20	2.893	0	0.16	0.696	0.44	0.28	0.566	0.226	2.033	62.83	29.48

3.3.6.3 MICROSCOPIC IMAGES OF CORRODED MILD STEEL COUPONS IN THE LOAM SOIL SYSTEM USING THE STEREO MICROSCOPE

Mild steel coupons immersed in a stimulated loam soil system were viewed using a stereo microscope at 4, 12 and 20 weeks of incubation. Fissures and pitting were observed on the surface of the mild steel coupons of the non-autoclaved sample at week 4 (arrow in Figure 3.9b), as compared to the autoclaved control (Figure 3.9a) that did not show much pitting. In the sample supplemented with sodium nitrate, pits were observed as well as the reddish-brown deposits present on the surface of the coupon (arrow in Figure 3.9c). In the sample treated with additional ammonium nitrate black-coloured substances were visible on the surface as well as a reddish-brown deposit (arrow in Figure 3.9d), which is characteristic of the main components, iron and oxygen (Table 3.4).

Product formation as a result of the corrosion process and rust formation was visible on the coupon surface in both the autoclaved and non-autoclaved controls as shown in Figure 3.10 (a and b). However, deep pitting was observed on the coupon surface of the non-autoclaved sample (arrow in Figure 3.10b). Pitting as well as deposit formation was observed on the mild steel coupon surface in the nitrate treated samples (Figure 3.10 c and d).

Severe corrosion was observed on the surface of the non-autoclaved sample (arrow in Figure 3.11b) at week 20 as compared to the autoclaved control (Figure 3.11a). An uneven and fissured surface layer was observed on the non-autoclaved coupon surface and in the samples with additional nitrates (Figure 3.11 c and d). The reddish-brown and black deposit formation was evident in the above-mentioned sample indicating severe corrosion (arrow in Figure 3.11d).

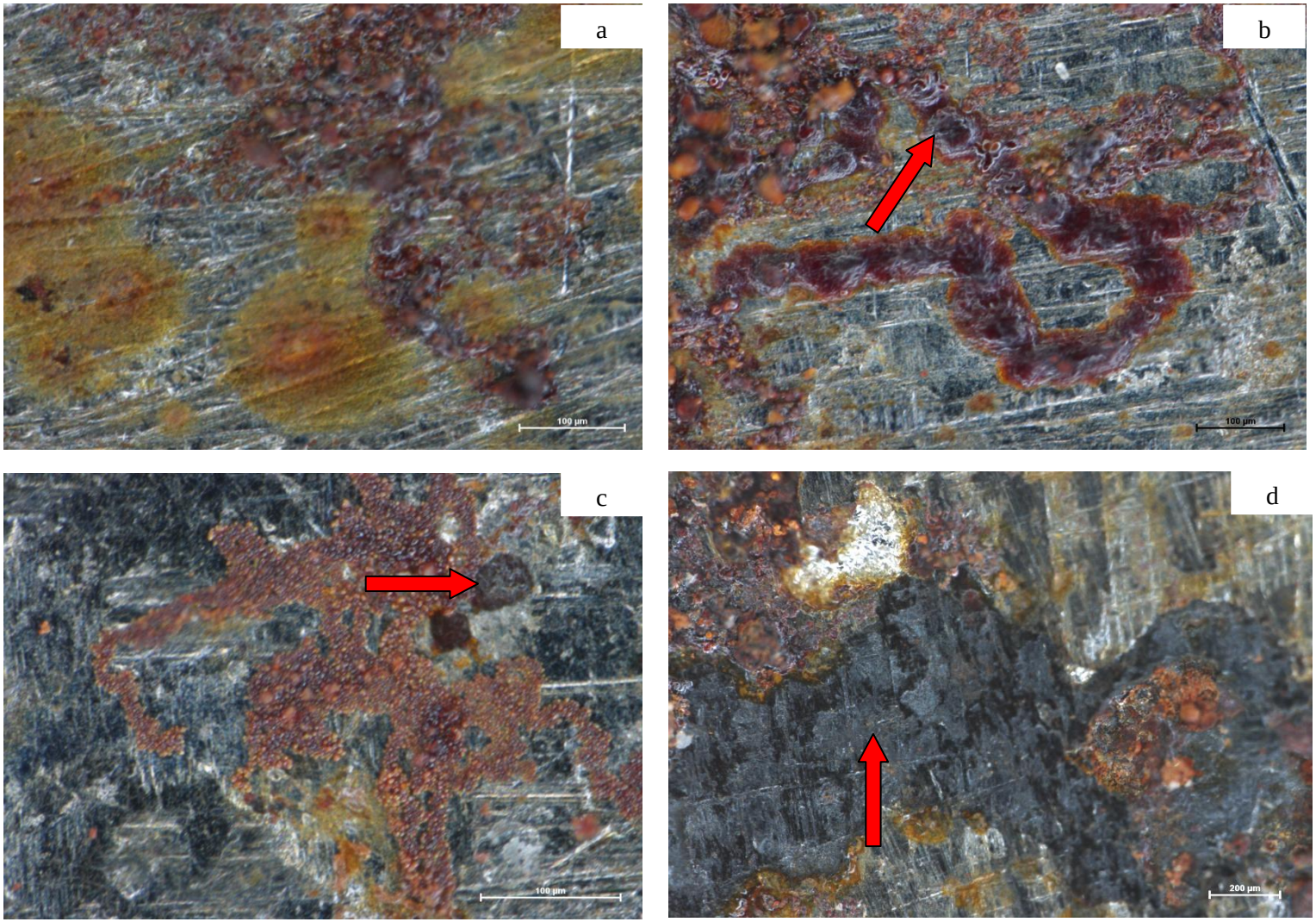

Figure 3.9: Stereo microscopic images of mild steel in loam soil after 4 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate (arrows indicate pitting and corrosion products)

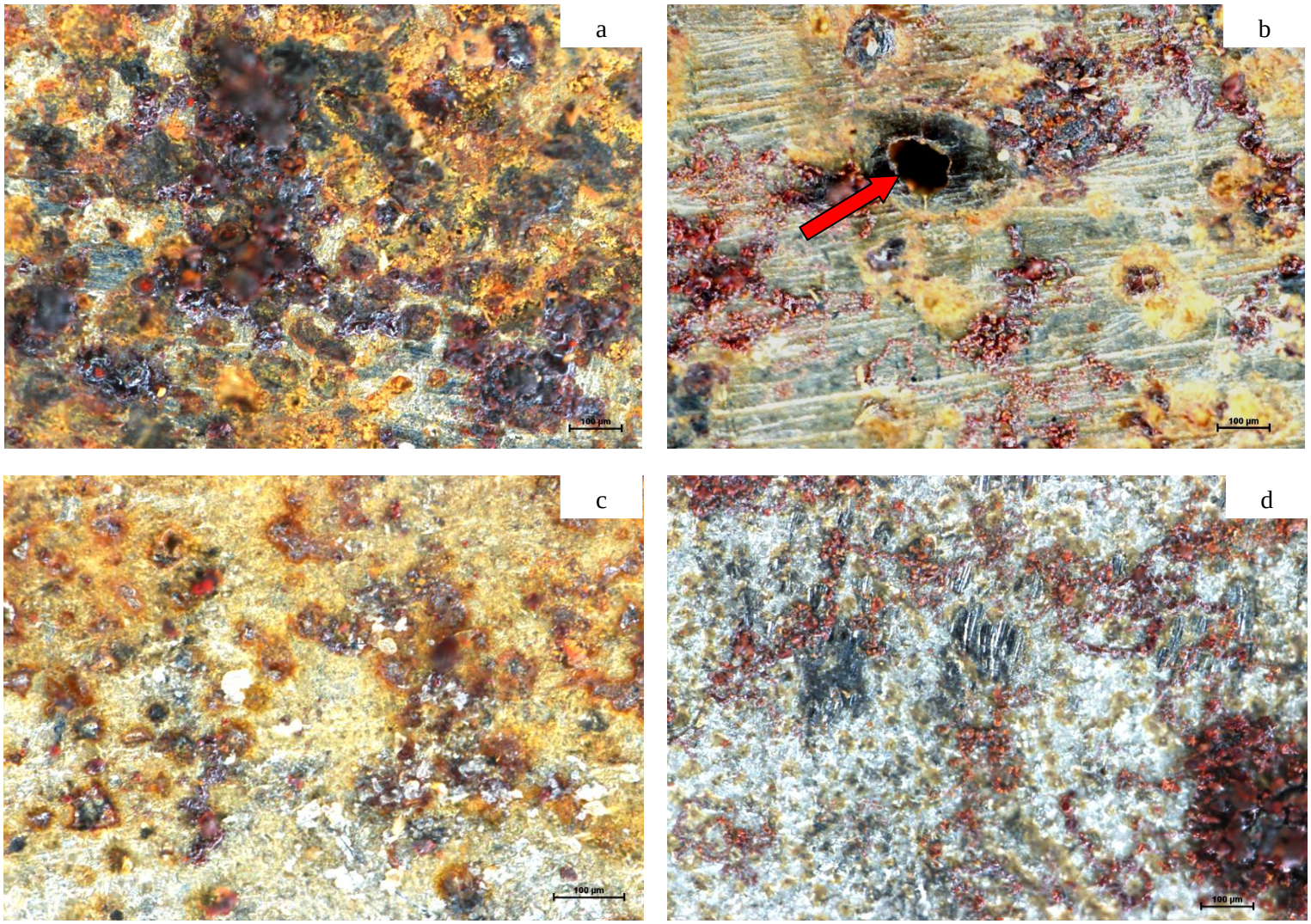

Figure 3.10: Stereo microscopic images of mild steel in loam soil after 12 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate (arrow indicate pitting and corrosion products)

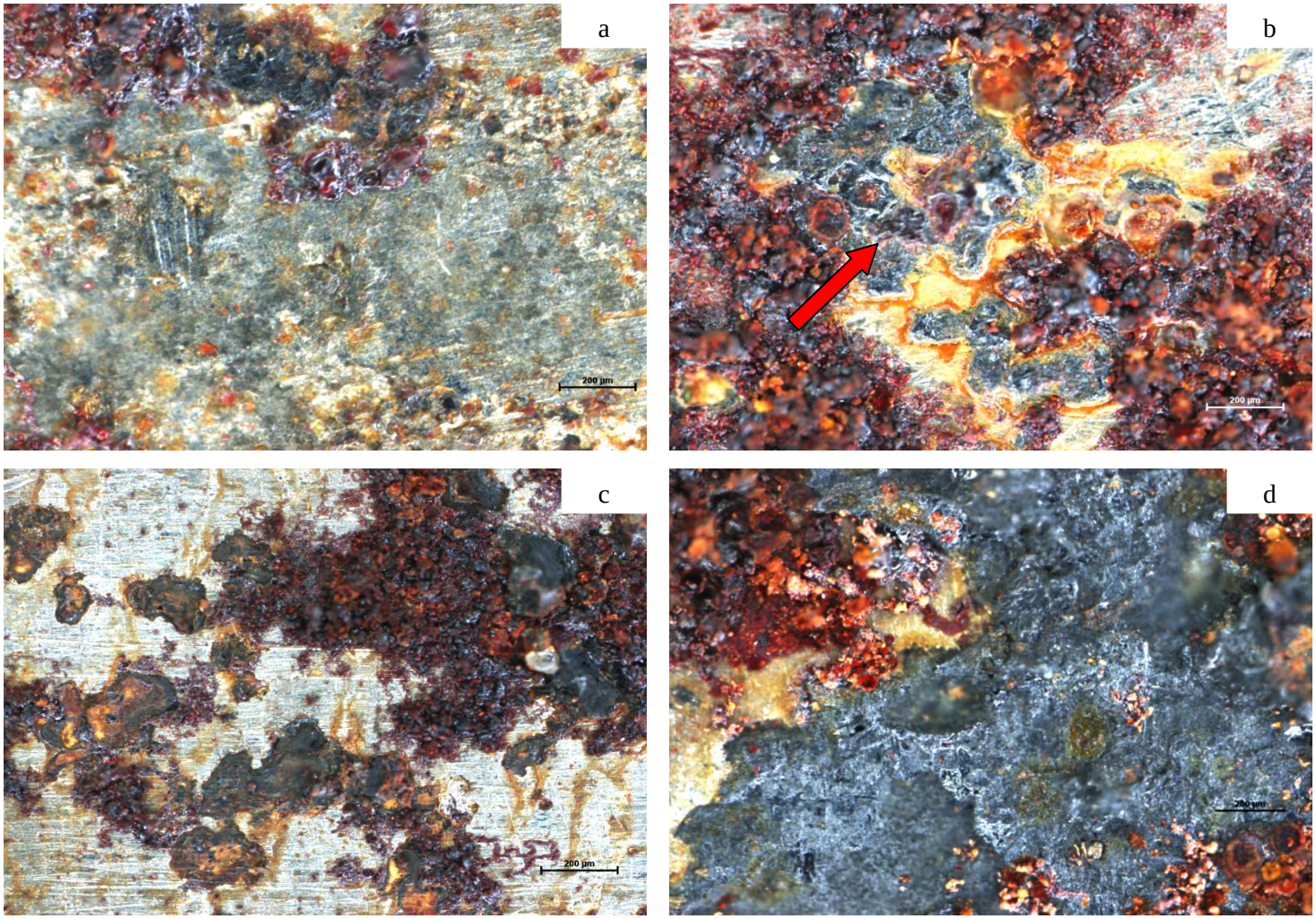

Figure 3.11: Stereo microscopic images of mild steel in loam soil after 20 weeks of incubation. (a) Control, (b) non-autoclaved system (c) system supplemented with sodium nitrate, and (d) system supplemented with ammonium nitrate (arrow indicate pitting and corrosion products)

3.4 DISCUSSION

Many complex chemical reactions take place on the steel surface when exposed to the environment. The type of rust, kinetics, mechanism and their stabilities are governed by factors such as the steels chemistry, type of environment, duration of exposure, etc. (Singh *et al*., 2008). Soil corrosion is caused by moisture, pH, redox potential, microbes in soils and soil type (Al-Sultani and Nabat, 2012; Ekine and Emujakporue, 2012). The results in this study indicate that the soil has a pH of 8.09, which is consistent with the range (4.5 to 9) specified by Costerton *et al*. (1995) for microbial activity to occur, though it is argued by Harris (1960) that pH within a range from 5 to 9 has no effect on corrosion.

In this study, lower rates of corrosion were detected on the stainless steel coupons in the nitrate-treated systems in loam soil as shown in Figure 3.1. The mild steel coupons in all nitrate treated systems (Figure 3.2) had similar weight loss measurements in both loam soil and the seawater/sediment system as discussed in Chapter 2. The addition of sodium nitrate had different effects on different metals, namely, there was a reduction in the rate of corrosion on stainless steel and acceleration in the corrosion rate on mild steel. In this case, the properties and composition of the metals as well as the physical characteristics of loam soil could also influence the process of corrosion. The composition of the metal surface available for bacterial colonization affects biofilm development and its metabolic activity. Bacterial attachment to the metal coupons may acquire some metallic elements present on the metal surface, thus influencing their development/activity (Lopes *et al*., 2006).

It was observed that after 20 weeks of incubation in the stimulated loam soil system, an increase in SRB and total heterotrophic bacterial population occurred in the mild steel samples supplemented with nitrates. A possible factor stimulating the rate of corrosion on the mild steel coupons could be that the additional nitrates stimulated bacterial growth (Gomez-Alvarez *et al*., 2012; Zakaria *et al*., 2012). Microscopy observations revealed microbial adherence and colonization with biofilm formation. The formation of needle-like corrosion products were observed on the mild steel coupons in the loam soil samples (Figure 3.7). SEM observations confirm the presence of microorganisms and this could be related to their role in the corrosion process. Many of the conclusions about biofilm development, composition, distribution, and relationship to substratum/corrosion production have been derived from traditional SEM (El Abed *et al*., 2012).

However, there are fundamental problems in attempting to diagnose MIC by establishing a spatial relationship between numbers and types of microorganisms in the bulk medium and those associated with corrosion products (Little *et al*., 2011). It was established that there were no relationships between the presence, type or levels of planktonic or sessile bacteria and the occurrence of pits. It is known that microorganisms are ubiquitous and therefore the presence of bacteria does not necessarily indicate a casual relationship with corrosion (Little *et al*., 2006).

In the nitrate treated loam soil systems, lower concentrations of proteins and carbohydrates were observed in the biofilms that formed on the mild steel coupons (Tables 3.2 and 3.3). Both the protein and carbohydrate concentration indicates their significance in biofilm development (Compere *et al*., 2001). Several studies have shown that bacteria produce lower concentrations of carbohydrate and higher amounts of protein during the early logarithmic phase of microbial metabolism associated with corrosion (Rice *et al*., 2000; D'Souza *et al*., 2005). According to D'Souza and Bhosle (2003), the carbohydrate and protein concentrations increase with longer exposure times. Similarly, they also observed relative differences in the distribution of carbohydrates and proteins in biofilm developed on mild steel, stainless steel and copper panels immersed in the surface seawater. However, in the present study, the concentration of both proteins and carbohydrates decreased with time upon exposure of mild steel coupons to the stimulated loam soil system.

EDX analyses on the mild steel coupons in the stimulated loam soil system indicated that the main elements of the corrosion products were iron and oxygen which is the same for that observed in the stimulated seawater/sediment system (Chapter 2). These elemental components are characteristic in the formation of the reddish-brown deposits seen in the stereo microscopic images of the corroded mild steel coupons (Figure 3.11). The stereo microscopic images allow visualization of the extent of corrosion that occurred on the mild steel coupon surface. An uneven surface with fissures and pitting was observed on the coupon surface indicating severe corrosion. The presence of the reddish-brown and black deposits indicates the possible activities and presence of iron-oxidizing bacteria and SRB that participate in the corrosion process (Chamritski *et al*., 2004; Xu *et al*., 2007). When the rate of oxygen diffusion into the biofilm is less than the rate of respiration, areas within the biofilm become anaerobic and SRB can proliferate in the locally anaerobic conditions beneath the biofilms such as the reddish-brown deposits produced by iron-oxidizing bacteria. Sulphide produced by the SRB then migrates to the edges of the deposits where it is oxidized to thiosulphate,

which is a well known activator of pitting corrosion (Angell and Urbanic, 2000; Xu *et al*., 2007). Black, sulphide-rich deposits are often found inside the iron-rich deposits formed by the iron-oxidizing bacteria. The interaction between SRB and iron-oxidizing bacteria accelerates the rate of corrosion (Xu *et al*., 2007).

Trace amounts of manganese, magnesium, silicon, aluminium and phosphorous were also detected in the corrosion products on mild steel coupons (Table 3.4). It is known that when the chromium (Cr) contents in the corrosion products are low, the concentration of chromium (III) oxide (Cr_2O_3) is insufficient for protecting the metal surface from corrosion attacks. Thus, the corrosion rate increases in the presence of bacteria (Ostwald and Grabke, 2004; Moradi *et al*., 2011). The potential mechanism of the effect of metal-oxidizing bacteria on the corrosion process can be explained by the fact that these microorganisms selectively remove some alloy elements, such as silicon, from the metal substratum. Organic and inorganic materials are deposited as corrosion products and metabolic materials on the metal surface, thereby increasing its susceptibility to pitting and crevice corrosion (Moradi *et al*., 2011).

The results obtained in the stimulated loam soil system corresponds with the results obtained in the stimulated seawater/sediment system. This indicates that the addition of nitrates in the form of sodium nitrate and ammonium nitrate stimulated the rate of corrosion on the mild steel coupons. The reduction in the corrosion rate on the stainless steel coupons observed in this study could be due to the resistant properties and metal compositions. However, with time, stainless steel is known to corrode as observed in Chapter two. It is known that corrosion rate measurements normally fluctuate with time. This is evident from the results obtained in the stimulated loam soil system. The degree of fluctuation can depend on the corrosion mechanism occurring. While corrosion is a dynamic process and subject to random variations even under constant conditions, the local environment is very sensitive to the microclimate in which they exist, in particular the moisture content (Law *et al*., 2004; Li *et al*., 2012). These results illustrate how important bacterial consortia and synergistic relationships between bacteria are to MIC. SRB are also contributors to MIC in not only anaerobic but also aerobic conditions. Microorganisms attach to surfaces and interact in complex ways to form biofilms and produce an environment at the biofilm/metal interface that is radically different from that of the bulk medium in terms of pH, dissolved oxygen, and organic and inorganic species (Xu *et al*., 2007; Malucknov, 2012).

As discussed in Chapter 2, environmental conditions pose as a major factor contributing to the corrosion processes of metals. In this study, it was noted that the corrosion process is different in the two environmental conditions due to the different microbial conditions present in the systems. As mentioned previously there are many influential parameters affecting corrosion in soils. These include the chemical content of soils which were not evaluated in this study. The knowledge of the content of the loam soil could have allowed optimization of the nitrate concentration for corrosion inhibition. Therefore, it is advisable to analyze the environmental type and composition before optimization of nitrate concentration that could lead to effective inhibition of corrosion on metal.

CHAPTER FOUR

MICROBIAL INFLUENCED CORROSION BY BACTERIAL SPECIES ISOLATED FROM CORRODED COUPONS: THE EFFECTS OF NITRATE SUPPLEMENTS

4.1 INTRODUCTION

In the industrial sector, microbes are thought to play an important role in the corrosion f metals such as iron and steel (Lin and Ballim, 2012). Corrosion can lead to potentially hazardous system malfunctions as well as costly damage and repair costs (El-Shamy *et al*., 2009). The rates at which various types of metals corrode are dependent upon environmental conditions including biotic and abiotic factors (Lin and Ballim, 2012). MIC has been defined as an electrochemical process in which the participation of microorganisms is able to initiate, facilitate or inhibit the corrosion reaction without changing its electrochemical nature (El-Shamy *et al*., 2009). Biocorrosion is the result of the synergetic interactions of the metal surface, abiotic corrosion products, bacterial cells and cell metabolites (Malucknov, 2012). Numerous reports have indicated that the most severe cases of local corrosion damage occurs due to the participation of microorganisms and may be responsible for 70 - 80 % of the total corrosion losses in the world (Nizhegorodor *et al*., 2008). MIC accelerates corrosive levels with the presence of a microbial consortium (Zuo, 2007) in which there is complex interactions between many physiological types of bacteria (Akpabio *et al*., 2011;Bano and Qazi, 2011). Microorganisms are known to influence the energy yielding corrosion reaction due to their opportunistic behaviour. This results in the corrosion process being enhanced in order to harvest the energy that has been released (Lin and Ballim, 2012).

The metal surfaces are mostly targeted by bacteria existing both in land and water and include the SRB, iron oxidizing/reducing bacteria, manganese-oxidizing bacteria and bacteria that secrete mucus and organic acids (Maluckov, 2012). Mechanisms by which bacteria corrode metals include the consumption of electrons from the elemental metal via cathodic polarization (Bano and Qazi, 2012). Microbial growth on the metal surface can promote the formation of corrosion cells with the metabolic end products being hydrogen sulphide which is very corrosive, accelerating corrosion and affecting the electrochemical processes (Lin and Ballim, 2012; Rajasekar *et al*., 2010; Zhao *et al*.,

2012). In the oil industry, hydrogen sulphide leads to reservoir souring as well as corrosion (Lin and Ballim, 2012). Heterogenous distribution of microorganisms on metal surfaces can cause the formation of differential aeration cells and, metal surface beneath the biofilms and minerals' precipitates acts as an anode, resulting in dissolution of the metal (Bano and Qazi, 2012).Sulphate-reducing bacteria are recognized as the major group involved in anaerobic corrosion. (). In particular, sulphur-oxidizing bacteria, methanogens and nitrate-reducing bacteria have been previously reported to be associated with metal biocorrosion (Bermont-Bouis *et al*., 2007).The sulphur oxidizers are capable of oxidizing elemental sulphur or sulphur-bearing compounds to sulphuric acid(Rothwell, 1979). The iron bacteria normally contribute to corrosion by creating differential oxygen concentrations and are capable of creating extremely corrosive conditions with the release of aggressive metabolites, such as acetic, succinic, isobutericor sulphuric acids (Coester and Cloeter, 2005).The stalked bacteria in the genus *Gallionella*, and filamentous bacteria in the genera *Sphaerotilus*, *Crenothrix*, *Leptothrix*, *Clonothrix*, and *Lieskeella* are also known as corrosionpromoters (Iverson, 1987; Coester and Cloeter, 2005).

Depositing dissolved organic matter onto metal surfaces, thereby conditioning the surfaces, is considered to be the first stage in biofilm formation. Conditioned surfaces are then colonized by microorganisms including bacteria, diatoms, fungi and protozoa(Barnes *et al*., 1999; Majumdar *et al*., 1999).Once a biofilm is developed on a material's surface, a microenvironment is created that is different from the bulk surroundings(Lane, 2005; Yuan *et al*., 2007; Akpabio *et al*., 2011). Bacterial biofilms produce differential aeration cells leading to localized changes in oxygen concentration, electrical potential and others (McNeill andEdwards, 2002), which increase corrosion rates (Coester and Cloeter, 2005; Lane, 2005; Yuan *et al*., 2007; Akpabio *et al*., 2011). Morales *et al*. (1993) and Franklin *et al*. (1991) found that the heterogenous biofilm of *Pseudomonas aeruginosa*formed on the metal surface may create differential aeration cells or metal ion concentration cells to cause the occurrence of pitting corrosion. However, biofilms produced by *Pseudomonas fragi*, *Escherichia coli*,*Bacillus brevis* and Actinomycetes may also serve as a barrier leading to surface protection against biocorrosion as shown by Garcia and Lopez (2012).

Corrosion inhibition can also be performed by substances such as corrosion inhibitors. When these substances are added in small quantities to a given environment, a decrease in the rate of corrosion on a metal is observed (Akpabio *et al*., 2011). Corrosion inhibitors are added compounds that decrease the corrosion reactions of metals (Sheng *et al*., 2012). An alternate corrosion control

strategy is nitrate supplementation or dosing that may work by different mechanisms (Marques *et al*., 2012. Established SRB activity can be controlled by nitrate addition which stimulates resident heterotrophic nitrate-reducing bacteria and nitrate-reducing, sulphide-oxidizing bacteria, collectively known as nitrate-reducing bacteria. The mechanism of control includes the production of nitrite, which is a strong SRB inhibitor, and the competition of heterotrophic nitrate-reducing bacteria and SRB for the same organic source (Grigoryan *et al*., 2008). In addition, some SRB may also switch their sulphate-based metabolism to nitrate reduction. However, nitrate supplementation has been shown to have different effects on the corrosion of carbon steel; it may either promote lower or higher corrosion rates (Marques *et al*., 2012). Previous studies demonstrated that nitrate or nitrite mediated control of corrosion has proved successful in combination with a NR-SOB culture dominated by *Thiomicrospira* sp. to a growing SRB consortium inhibited sulphide production (Hubert *et al*., 2003; Rempel *et al*., 2006).These studies observed that nitrate addition alone did not impose an inhibitory effect but did stimulate the NR-SOB metabolic activities leading to sulphide removal (Nemati *et al*., 2001b; Rempel *et al*., 2006).

A proper understanding of the identity and role of microbial contaminants present on the metal surface in the specific environments may be exploited to induce corrosion inhibition by bacteria as a useful tool to prevent frequent MIC effects encountered in practice. The results of the previous two chapters indicate that the supplementation of nitrates did not inhibit the corrosion processes in the seawater/sediment and in the loam soil environments. In this chapter, the bacteria were isolated from the corroded steel coupons in the above-mentioned environments. Sequence data analyses confirm identity of these microorganisms and phylogenetic analyses were used to identify similarity. The effect of nitrates on the corrosion of mild and stainless steel coupons, with isolated microorganisms individually and in consortia, was tested.

4.2 MATERIALS AND METHODS

4.2.1 EXPERIMENTAL SYSTEM SET-UP

The seawater/sediment system and the loam soil system were constructed according to Orfei *et al.* (2006) as described in Chapters 2 and 3 respectively. Mild steel and stainless steel coupons were treated and pre-weighed before immersion into the experimental systems as described in Chapter 2. After 2, 4, 8, 12, 16 and 20 weeks of incubation, duplicate stainless and mild steel coupons were

extracted from the respective experimental systems and were used to isolate bacteria as described in Section 4.2.2 below.

4.2.2 ISOLATION OF MICROBIAL ISOLATES FROM THE CULTIVABLE FRACTION OF THE BIOFILM FORMED ON THE CORRODED METAL SURFACE

The coupons extracted from the respective experimental systems were swabbed (25 mm Gamma sterilized cotton swabs) and spread plated onto nutrient agar plates. The agar plates were incubated at 30°C overnight or until bacterial growth was observed. Purification of the isolates was achieved by streaking on nutrient agar plates. Once pure isolates were obtained, these were subjected to Gram staining and spore staining according to the standard methods (Dhanasekaran *et al*., 2009; Correia *et al*., 2010) and 16S rRNA identification (Marchesi *et al*., 1998).

4.2.3 COUPONS AND INOCULUM PREPARATION

Stainless steel and mild steel coupons were pre-treated and pre-weighed as described in Section 2.2.1 of Chapter 2. The inoculum was prepared by growing the bacterial cultures in 30 ml nutrient broth at 37 °C on a rotary shaker at a speed of 150 rpm for 24 hrs. The isolates were centrifuged using the Beckman Coulter, Avanti, J-26 XPI centrifuge at 4000 rpm for 20 mins. The subsequent pellet was washed three times with 0.85 % (w/v) saline solution and resuspended in a smaller volume of 0.85 % (w/v) saline solution. The inoculum was standardized to a final absorbance of approximately 1.5 at an optical density of 600 nm.

4.2.4 EVALUATION OF CORRODING ACTIVITIES OF DOMINANT MICROORGANISMS ISOLATED FROM BIOFILMS

Three sets of experiments were conducted to determine the corroding activities of the isolated microorganisms. The coupons were immersed in a vertical position in 250 ml Erlenmeyer conical flasks containing 100 ml of sterilized deionized Millipore water (Millipore Elix purification system, 17 mΩ) or of 0.5 mM ammonium nitrate/sodium nitrate solution as the controls. One percentage (v/v) of standardized bacterial culture was inoculated into three respective solutions. The flasks were incubated at 30°C and subjected to agitation (100 rpm) in order to obtain a homogenous

solution. After two-week and three-month incubation for mild steel and stainless steel coupons respectively, weight losses of the mild steel and stainless steel coupons were measured as described in previous chapters. The effect of microbial consortia (equal concentration of each isolate was used to obtain a final volume of 1 %) on the corrosion rate of mild steel coupons was also evaluated under the same setups and conditions of each individual isolate. However, the effect of consortia was not evaluated on stainless steels due to the slow rate of corrosion that occurs.

4.2.5 DNA EXTRACTION

DNA was isolated from the pure isolates using the boiling-centrifugation method (Freschi*et al*., 2005). Template DNA was prepared by inoculating a loopful of culture from nutrient agar plates into an Eppendorf tube containing 400µl of sterile distilled water. The sample was placed in a water bath for 10 mins at 100°C. The mixture was then centrifuged at 13000rpm for 5 mins. DNA in the supernatant was used as the template for PCR amplification.

4.2.6 16S rRNA GENE AMPLICATION AND SEQUENCING

The reserved region of 16S rRNA gene (1386 bp amplicon) was amplified by PCR from the DNA extracted from isolated colonies. The forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') were used for this purpose (Marchesi *et al*., 1998). The reaction mixture for PCR amplification contained 1µl each of forward and reverse primers (50 pmol μl^{-1} each), 25 µl ReadyMix Taq PCR Reaction Mix with $MgCl_2$(2x) (Fermentas Life Sciences)and 1 µl of template DNAfrom each isolate was used. The composition of the PCR Master Mix (2x) is 0.05 U/µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP. PCR amplification was achieved using conditions according to Sambrook*et al*. (1989), using the ThermoHybaid PCR Express Thermal Cycler (Ashford, Middlesex) under the following cycling parameters: 30 cycles of initial denaturation at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 mins, followed by the final elongation step at 72°C for 5 mins (1 cycle). The PCR products were evaluated electrophoretically on a 1% (wt/vol) agarose gel at 80V for 90 mins in 1x Tris-Acetate – EDTA running buffer, stained with ethidium bromide and visualized with the Chemi Genius^2 BIO Imaging System and Gene Snap software (Syngene, UK). Product size was determined using Generuler DNA ladder mix (Fermentas).

4.2.7 DATABASE SEARCHES AND PHYLOGENETIC ANALYSIS

Bacterial isolates were identified by the analysis of their 16S rRNA amplified gene sequences.Amplified products were sequenced by Inqaba Biotech (South Africa). BLAST searches were performed throughout the Genbank databases using National Center for Biotechnology Information (NCBI) BLAST (http://www.ncbi.nlm.nih.gov/BLAST) comparison software (Altschul *et al*., 1990; Altschul *et al*., 1997). Sequences were aligned with the closest matching 16S rRNA gene sequences. A 97-100 % match of the unknown DNA sequence with the Genbank dataset was considered as an acceptable identification at the species level. The most similar sequences obtained by database searches were used to construct multiple alignments using the Mega4 (Tamura *et al*., 2007). The sequences were submitted to GenBank to obtain valid accession numbers.Thereafter, phylogenetic relationships were thus determined using the neighbour-joining bootstrap method using the Mega 4 software (Tamura *et al*., 2007).

4.2.8 STATISTICAL ANALYSIS

Paired t-tests (Wilkinson, 1988) were used to examine the statistical significance between the different treatments with pure isolates and their consortia on the rate of corrosion on mild steel coupons. Probability was set at < 0.05.

4.3 RESULTS

Based on the Gram staining results and cultural characteristic similarities, a total of 26 and 20 pure isolates were obtained from the corroded stainless steel and mild steel coupons respectively. The corrosion abilities of these isolates were evaluated using metal coupons under various conditions.

4.3.1 INVESTIGATION OF THE CORROSION PROCESS WITH THE ISOLATED MICROORGANISMS

After two weeks of incubationno weight loss was observed when the mild steel coupons were immersed in the deionized water (Figure 4.1; control 1). However, adding sodium nitrate and ammonium nitrate to the deionized solution stimulated an increase in the weight loss measurements of mild steel coupons to 26.89 mg/g and 26.83 mg/g respectively, after the same incubation period

(Figure 4.1). There were significant increases ($p < 0.05$) in the levels of corrosion (up to 150 mg/g mild steel coupon) due to the presence of bacterial isolates compared with the controls. A significantly ($p < 0.05$) lower weight loss (35.59 mg/g) was observed in the sample inoculated with Isolate LMS2 alone compared with all other isolates. The results indicate that Isolate LMS2 has less capacity to stimulate the corrosion process of mild steel coupons under the experimental conditions. The addition of ammonium nitrate with some bacterial isolates (CMS1, NMS1, NMS3, SMS2, SMS4, NML2, NML51, LMS21, LMS3 and LMA1) reduced the weight loss of the mild steel coupons as compared to the weight loss measured with the pure isolates alone (Figure 4.1). The results obtained show that the supplementation of nitrates effectively retarded the metal corrosion process that was provoked due to the presence of the bacterial isolates under the experimental conditions.

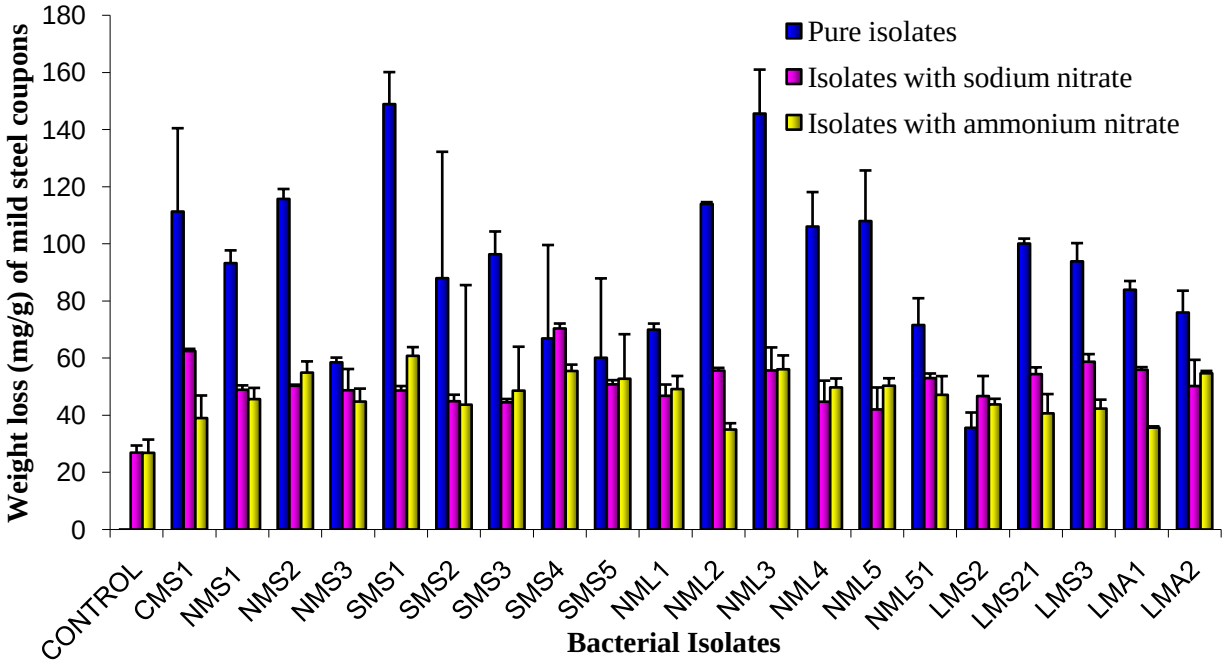

Figure 4.1: Weight loss of mild steel coupons due to microbial isolates with or without nitrate addition after 2 weeks of incubation at 30 °C, 100 rpm. The control: without bacterial isolate

The weight losses of stainless steel coupons were measured after three months of exposure in sterilized deionized water with bacterial inocula with or without additional nitrates. The results are shown in Figure 4.2. Weight losses of stainless steel coupons after three months were significantly ($p < 0.05$) less compared to the weight loss observed on mild steel coupons after two weeks, as

shown in Figure 4.1. Inoculating Isolates LS007, CL002, CS001, LA002, LA003, SA002, SA003 and SA004 demonstrated no weight loss of the stainless steel coupons in deionized water. The maximum weight loss (0.15 mg/g) of stainless steel coupons was observed when Isolate CS002 was supplemented together with ammonium nitrate in the medium. The presence of sodium nitrate supplementation did not affect the weight loss of the stainless steel coupons with the presence of Isolates NL001, NL002, LS005, LA003, SA003 and SA004. The addition of ammonium nitrate demonstrated a significant ($p < 0.05$) reduction in weight loss with Isolates SA001 and LS004 (0.03 mg/g and 0.02 mg/g respectively) as compared to weight losses observed with the pure isolates individually and isolates supplemented with sodium nitrate. The supplementation of sodium nitrate significantly ($p < 0.05$) reduced the weight loss of stainless steel coupons in the corrosion assay media with Isolates CS001, CS002, CL001, CL002, NL003, LS004, LS007, LS008, LA003, SS001, SS002, SA001, SA002 and SA005 (from 0.008 mg/g to 0.053 mg/g) as compared to the respective weight losses observed with the supplementation of ammonium nitrate.

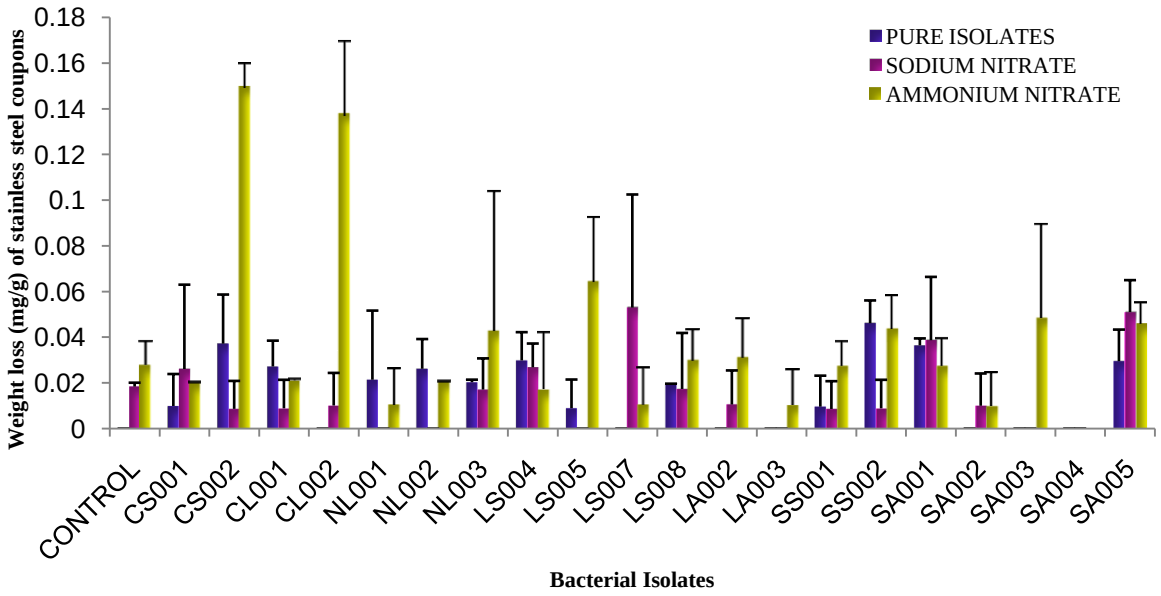

Figure 4.2: Weight loss of stainless steel coupons due to microbial isolates with or without nitrate addition after 3 months of incubation at 30 °C, 100 rpm. The control: without bacterial isolate

The weight loss of mild steel coupons was measured using a consortia of the microorganisms isolated in this study, as shown in Figure 4.3. In order to search for the bacteria that are capable of reducing weight loss under the study conditions, eight different isolates, which exhibited reduced weight loss in Figure 4.1, were used to develop a microbial consortium. Increased rates of corrosion

were observed in the sample with the consortia LMS2 and NMS3 (69.55 mg/g) compared to all the samples and controls however, with the addition of nitrates, the same bacterial consortia (LMS2 and NMS3) demonstrated a significant ($p < 0.05$) decrease in weight loss on mild steel (55.5 mg/g and 40.25 mg/g for sodium nitrate and ammonium nitrate supplementation respectively) as compared to the consortium control. The addition of ammonium nitrate to the assay medium demonstrated a significant ($p < 0.05$) increase in weight loss (44.15 mg/g) as compared to the control with no nitrates or isolates (1.3 mg/g). A decrease in weight loss of the coupons was observed with the consortium LMA1 + LMS21 + ammonium nitrate (40.9 mg/g) as compared to the weight loss observed with the pure isolates, as shown in Figure 4.1 (LMS21 and LMA1 displayed 100 mg/g and 83.925 mg/g weight loss respectively). The addition of the consortium CMS1 + LMA1 with ammonium nitrate addition seem to effectively reduce the weight loss of the coupons (40.35 mg/g) as compared to that observed with the pure isolates in Figure 4.1 (CMS1 and LMA1 displayed a weight loss of 111.295 mg/g and 83.925 mg/g respectively). The consortium SMS2 + CMS1 with sodium nitrate addition displayed a weight loss measurement of 53.15 mg/g of the coupons as compared to the measurements observed in Figure 4.1 with the pure isolates (87.97 mg/g and 111.295 mg/g for SMS2 and CMS1 respectively). The supplementation of sodium nitrate to the consortium SMS2 + SMS3 displayed a reduced weight loss measurement of 47.45 mg/g compared to the pure isolate inoculation (Figure 4.1) that displayed weight loss measurements of 87.97 mg/g and 96.33 mg/g for SMS2 and SMS3 respectively.

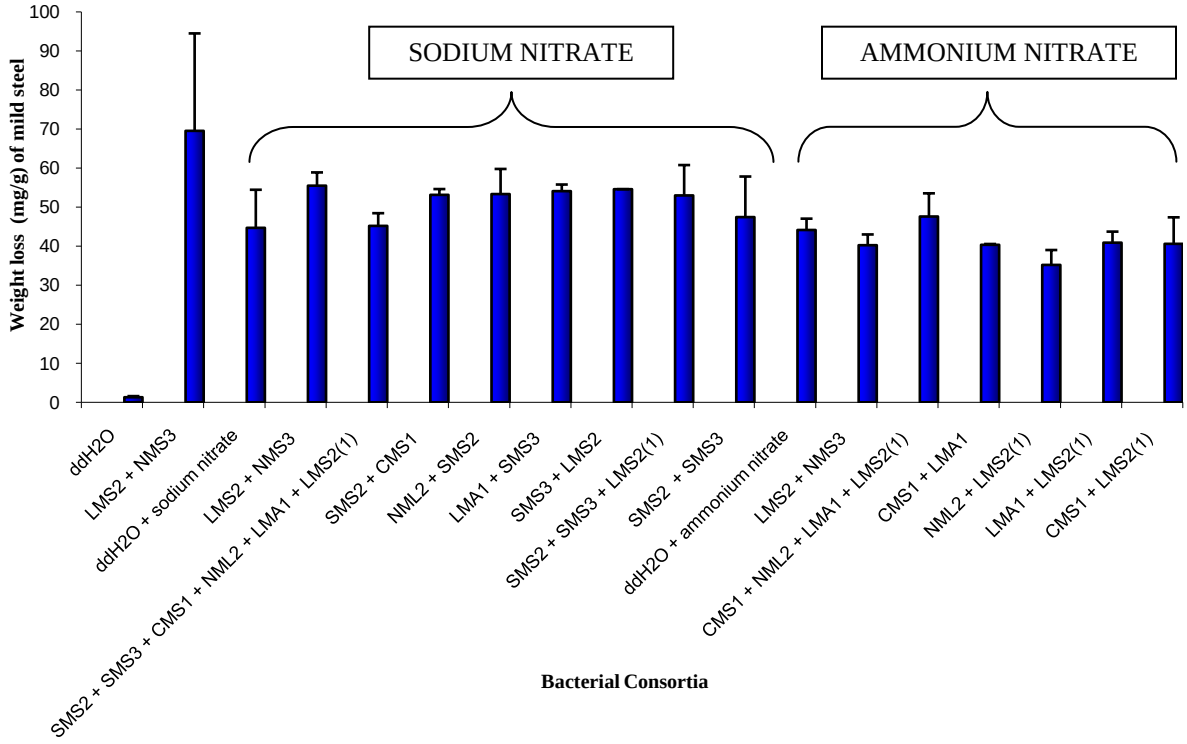

Figure 4.3: The effect of microbial consortia and nutrient addition on the corrosion of mild steel after 2 weeks of incubation

4.3.2 SEQUENCE ANALYSES OF ISOLATED MICROORGANISMS FROM THE STEEL COUPONS

The identities of 12 and 13 isolates extracted from both mild and stainless steel coupons respectively, were successfully confirmed using 16S rDNA sequencing (Tables 4.1 and 4.2). The 16S rDNA sequence data analysis established that 20 of the isolated microorganisms from both corroded mild steel and stainless steel coupons belongs to the genus *Bacillus* and is affiliated to the bacterial phyla *Firmicutes*. Isolates LMS21 and LMS1 (Table 4.1), which were isolated from mild steel buried in loam soil with additional sodium nitrate, revealed a 99 % similarity to *Pseudomonas* sp. belonging to the bacterial phyla *γ-Proteobacteria.* Isolates SA003 and SA005 were isolated from stainless steel coupons (Table 4.2) immersed in the seawater/sediment system stimulated with ammonium nitrate, revealed a 99 % and 100 % similarity to *Marinobacter* sp. respectively. Isolate SA004 (Table 4.2) revealed a 99 % similarity to *Halomonas* sp. These three isolates from corroded stainless steel coupons belong to the subdivision *γ-Proteobacteria.*

Table 4.1: Summary of the bacterial identifications based on 16S rDNA sequences from the mild steel coupons in this study

Affiliation	**16S rDNA Sequence (GenBank Accession No.)**	**Closest Relative according to Blast search (Accession No.)**	**% of identity with the closest relative**
Firmicutes	LMA1 (JN106424)	*Bacillus megaterium* (HQ647284)	99 %
γ-Proteobacteria	LMS21 (JN106425)	*Pseudomonas* sp. (EU841539)	99 %
Firmicutes	LMA2 (JN106404)	*Bacillus* sp. (HM566526)	99 %
Firmicutes	LMS2 (JN106405)	*Lysinibacillus* sp. (HM057828)	99 %
Firmicutes	LMS3 (JN106406)	*Bacillus sp.* (JF742977)	100 %
Firmicutes	NML2 (JN106410)	*Bacillus sp.* (JF768715)	100 %
Firmicutes	NMS3 (JN106411)	*Bacillus cereus* (JF714217)	99 %
Firmicutes	SMS3 (JN106416)	*Bacillus megaterium* (HQ647278)	99 %
Firmicutes	SMS2 (JN106419)	*Bacillus aquimaris* (JN559392)	100 %
Firmicutes	NML1 (JN106420)	*Bacillus megaterium* (HQ242768)	99 %
γ-proteobacteria	LMS1 (Accession number not available)	*Pseudomonas* sp. (JN129432)	99 %
Firmicutes	SMS1 (Accession number not available)	*Bacillus sp.* (JQ030918)	99 %

Table 4.2: Summary of the bacterial identifications based on 16S rDNA sequences from the stainless steel coupons in this study

Affiliation	**16S rDNA Sequence (GenBank Accession No.)**	**Closest Relative according to Blast search (Accession No.)**	**% of identity with the closest relative**
Firmicutes	CL002 (JN106423)	*Bacillus aerophilus* (JF496287)	100 %
Firmicutes	CS001 (JN106426)	*Bacillus pumilus* (HM103345)	99 %
Firmicutes	LA003 (JN106427)	*Bacillus* sp. (JF768714)	99 %
Firmicutes	LS007 (JN106407)	*Bacillus idriensis* (JF496378)	99 %
Firmicutes	NL003 (JN106408)	*Bacillus megaterium* (HQ647284)	99 %
Firmicutes	LS004 (JN106409)	*Bacillus megaterium* (EU221370)	99 %
Firmicutes	SA001 (JN106412)	*Bacillus sp.* (GU084178)	100 %
γ-proteobacteria	SA003 (JN106413)	*Marinobacter* sp. (FJ903190)	99 %
γ-proteobacteria	SA004 (JN106414)	*Halomonas* sp. (AB305227)	99 %
γ--proteobacteria	SA005 (JN106415)	*Marinobacter* sp. (FJ903190)	100 %
Firmicutes	SS001 (JN106417)	*Bacillus aerophilus* (JF496287)	99 %
Firmicutes	SS002 (JN106418)	*Bacillus* sp. (HM462445)	100 %
Firmicutes	LA002 (JN106422)	*Bacillus thuringiensis* (JN205290)	100 %

4.3.3 PHYLOGENETIC ANALYSES OF THE ISOLATED MICROORGANISMS

A phylogenetic tree (Figure 4.4) was constructed from distances calculated using the Maximum Composite Likelihood method and the Neighbor-Joining method. Eukaryotic *Amorphotheca resinae* and *Penicillium* sp. were chosen as outgroups.The majority of the isolated *Bacillus* species are placed within a branch with support (47 % bootstrap). The *Marinobacter* sp. (SA003 and SA005) and *Halomonas* sp. (SA004) formed a clade with 89 % bootstrap support within the second branch. Support for these two branches was strong showing uniform support for that clade (89 % bootstrap).

Bacillus megaterium LMA1 and *Pseudomonas* sp. LMS21 were represented in another major branch with good support (99 % bootstrap)and considered to be statistically significant.

Figure 4.4: The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 458 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.(*indicates microorganism isolated in this study)

4.4 DISCUSSION

The results of the previous two chapters indicate that the supplementation of nitrates did not inhibit the corrosion processes in the seawater/sediment and in the loam soil environments. The results from this study show that the individual isolates provide an oxidation-reduction tool for metal corrosion especially for the mild steel coupons. The addition of sodium nitrate and ammonium nitrate reduced the corrosion rate of mild steel coupons induced by the respective bacteria isolated in this study (Figure 4.1). However, nitrate addition demonstrated different effects on the weight loss measurements on the stainless steel coupons (Figure 4.2). In some cases it was demonstrated that the addition of ammonium nitrate with Isolate CS002 promoted an increase in weight loss of stainless steel coupons and with Isolates SA001 and LS004, there was a decrease in weight loss observed. However, addition of sodium nitrate did not have any effect on the weight loss measurements of the coupons with some bacterial isolates.

This study correlates with a previous study (Jack *et al*., 1992) which observed that monocultures of isolated organisms developed a biofilm and induced greater corrosion rates in the initial stages of the study. There was a more significant reduction in weight loss of the stainless steel coupons than the mild steel coupons. This is mainly due to the elemental composition (as described in previous chapters) of stainless steel coupons, making them more resistant to corrosion than mild steel coupons. Stainless steels are known to be corrosion resistant alloys with high toughness and mechanical strength with excellent alloy elements (Cr, Mo, N and Ni) that renders this metal somewhat resistant to corrosion (Tavares *et al*., 2007).

MIC is normally caused by the physical presence and attachment of microbial cells onto the surfaces. One possible explanation is that fewer bacterial attachments on the surface results in less localized corrosion, because the attached microbial cells directly accept the electrons from the metal to cause corrosion (Sheng *et al*., 2012). It has been reported that nutrients play a very important role in enhancing the production of bacterial metabolites and the inhibition of corrosion (Rajasekar and Ting, 2011). The effect of nitrates and microbial consortia on mild steel corrosion was also observed. It was observed that certain consortia of microorganisms reduced the weight loss measurements of the coupons than that observed with the individual isolates. However, this depends on the isolates' individual capability of weight loss reduction. From the results obtained, it can be observed that in some cases ammonium nitrate supplementation to the assay media with certain

consortia reduced the weight loss of the mild steel coupons (Figure 4.3). Therefore, it seems that further experiments need to be conducted to further optimize nutrient and inoculum concentrations for optimum corrosion inhibition. These results simply provide the basis of the potential of these isolates in reducing the weight loss of mild steel coupons. This decrease in weight loss measurements could be attributed to microbial respiration of the individual isolates in a consortium, resulting in a decline of oxygen concentration at the metal surface and an associated decrease in the rate of the cathodic reduction of oxygen (Lewandowski and Beyenal, 2008). It was previously speculated that the provision of nutrients might have created a more or less continuous biofilm across the metal surfaces and played a role in reducing the corrosion process (Bano and Qazi, 2011).

Bacteria can protect the metal by several different processes such as 1) passive film formation, 2) the presence of a uniform layer of living aerobic bacteria whose respiration decreases oxygen concentration, 3) formation of a diffusion barrier, as a result of exopolymers produced by attached bacteria, and 4) production of antimicrobial compounds or corrosion inhibitors (Lewandowski *et al*., 2003). The microbiological component of corrosion is a very complex process and is seldom identified as a single organism or a unique mechanism. Marques *et al*. (2012) suggested that nitrates regulated community complexity, most likely by stimulating bacterial competition. However, it was further proposed that the nitrate effects on total bacterial structure did not lead to strong changes in these communities. Higher corrosion rates using nitrate supplementation to control corrosion was observed in the previous chapters and in previous studies (Nemati *et al*., 2001a; Hubert *et al*., 2005; Marques *et al*., 2012). This was effectively seen in Figure 4.2, whereby the addition of ammonium nitrate increased the weight loss measurements on stainless steel coupons in the presence of some bacterial isolates.The difference in corrosion measurements of the metal coupons observed in the previous chapters and that with the bacterial isolates could be due to the environmental conditions harboring a complex biological system. In this chapter, the bacterial isolates were grown in a nutrient rich medium that probably altered their metabolic capabilities as compared to their activity in the seawater/sediment and loam soil systems. Another contributing factor is that there are many unculturables within the environments tested in the previous chapters, thereby limiting the number of bacterial species isolated for the particular study described in this chapter. Due to this limitation, a molecular approach was taken in the next chapter in order to detect more bacterial isolates involved in the corrosion process.

Isolation, identification and sequence analysis on the microorganisms revealed that the majority were of the *Bacillus* sp. affiliated with the bacterial phyla *Firmicutes* (Tables 4.1 and 4.2). The influence of *Bacillus* sp. on corrosion of steel has also been reported by several workers (Sreekumari *et al*., 2004; Matsumura *et al*., 2007). In addition, *Pseudomonas* sp. (JN106425 and JN129432), *Marinobacter* sp. (JN106413 and JN106415), and *Halomonas* sp. (JN106414) were identified and these were affiliated with the bacterial phyla γ-*Proteobacteria*. Stainless steel surfaces are also generally hydrophobic (Kang and Lee, 2011) and hence it appears that the high hydrophobicity of the *Bacillus* sp. favours adhesion to stainless steel surfaces (Anandkumar *et al*., 2011). Bacteria forming biofilms are well known to protect different kinds of metals by consuming dissolved oxygen and making it inaccessible to the corrosion action. Secretion of some antimicrobial substances by the *B. thuringiensis*-SN8 might have been responsible for the observed corrosion protection effects (Bano and Qazi, 2011). These antimicrobial substances can be small molecules, which are structurally diverse, including bacteriocin and exoenzymes, like proteases, RNA-degrading enzymes, cell wall lytic enzymes and amylases. Some of these substances are active only against the same and closely related species, while others have a broad spectrum activity (Bano and Qazi, 2011). The Isolate LA002 (JN106422), *B. thuringiensis* used in this study did not affect the weight loss measurements on stainless steel coupons (Figure 4.2). However, with the addition of nitrates, an increase in weight loss measurements was observed. It could be possible that this isolate, when inoculated as a pure culture, displayed some protective mechanism on the steel but the additional nitrates might have increased its corrosive capabilities.

Iron-oxidizing microorganisms are capable of depositing iron hydroxides (rust) extracellularly at a rate of hundreds time higher than the abiotic process. Consequently, iron oxidizing and iron precipitating bacteria are among the most dangerous microorganisms in the biofouling and corrosion processes (Starosvetsky *et al*., 2008). These bacteria gain energy through the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), which occurs at near neutral pH and may result in the formation of iron oxides (Lin and Ballim, 2012). These organisms create conditions favourable to localized corrosion, especially in the case of metals passivity. Iron-oxidizing bacteria are micro-aerophillic and therefore are found commonly associated with other microorganisms in aerobic environments in the presence of reduced iron (Coetser and Cloete, 2005). Ferric ions present in the environment can serve as strong oxidizing species which may accelerate corrosion (Lin and Ballim, 2012). Dense deposits of biomass layer produced by the iron oxidizing bacteria on metal surface

provide conditions for the development of crevice effect on metal/biomass interface initiating pitting, thereby accelerating the corrosion process (Starosvetsky *et al*., 2008).

Phylogenetic relationships (Figure 4.4) were analyzed between the 16S rDNA sequences of the bacterial phyla *Firmicutes* and *Proteobaceria* in the phylogenetic trees. The phylogenetic analysis of the isolates showed the dominance of *Bacillus* sp. which is also in accordance with the study conducted by Rajasekar *et al*. (2010). Bacteria belonging to the genera *Bacillus* have been previously found associated with corrosive biofilms in pipelines transporting water or petroleum products (Lopez *et al*., 2006; Rajasekar *et al*., 2007b and Rajasekar *et al*., 2010). *Bacillus* sp. are suggested to be the primary colonizers in biofilm formation and are well-known exopolysaccharide and organic acid producers that accelerate steel corrosion (Marques *et al*., 2012). These bacteria are Gram-positive, spore-forming bacteria that are able to produce metal oxides at low pH and thus accelerate the pitting of a steel surface (Rajasekar *et al*., 2010). Isolates SMS1 and NML2 identified as *Bacillus* sp. were observed to increase the weight loss measurements of mild steel coupons when inoculated as pure isolates in the assay medium as shown in Figure 4.1. In addition, Isolates SS002 and CL001 identified as *Bacillus* sp. and *Bacillus pumilus* respectively, showed similar corrosive abilities on stainless steel coupons as shown in Figure 4.3.

Pseudomonas sp. contribute to biofilm formation by producing EPS that facilitate attachment of other microorganisms and thus accelerate the corrosion process (Oliveira *et al*., 2011). In Figure 4.1, the Isolate LMS21, *Pseudomonas* sp., demonstrated an increase in weight loss of the mild steel coupons when inoculated as a pure isolate however, the addition of nitrates reduced the weight loss measurements in the assay medium. This could be attributed to the nitrates providing the nutrients to the isolates, thus reducing their corrosive attack on the metal itself. In a previous study by Obeukwe *et al*. (1987), a *Pseudomonas* sp. was shown to accelerate corrosion with the addition of a carbon source suggesting bacterial activity correlated with MIC. *Bacillus* and *Pseudomonas* sp. have also been reported to be manganese oxidizers and to influence corrosion on stainless steel (Sreekumari *et al*., 2004). Rajasekar and Ting (2011) concluded that MIC by *Pseudomonas* sp. and *B. megaterium* on stainless steel SS304 inhibited the production of EPS and the passivity of SS304. They further suggested that nutrients play an important role in enhancing the production of bacterial metabolites and corrosion inhibition.

The *Halomonas* and *Marinobacter* groups are potential examples of opportunistic microbes (Kaye *et al*., 2011). For example, physiological cultivation-based studies have demonstrated that these organisms are neither metabolic specialists nor minimalists but rather follow a strategy of acquiring and maintaining broad functional potential to exploit spatially and temporally variable resources (Singer *et al*., 2011). Most *Marinobacter* species are aerobic although some members play a role in the denitrification process. They are halophilic and are commonly associated with seawater. Nevertheless, these bacteria may corrode steel with or without nitrate addition (Yakimov *et al*., 2005; Yakimov *et al*., 2007; Marques *et al*., 2012). However, in this study, Isolates SA003 and SA004 (*Marinobacter* sp. and *Halomonas* sp. respectively) demonstrated no weight loss of the stainless steel coupons in the assay media with the pure isolate and with the addition of sodium nitrate (Figure 4.2). Previous studies have shown that *Marinobacter* and *Halomonas* sp. have been grown with hydrocarbons under nitrate-reducing conditions (Bonin *et al*., 2002; Larsen *et al*., 2006), and *Marinobacter* spp. have been isolated from oil fields including those undergoing nitrate treatment (Sette *et al*., 2007). Dunsmore *et al*. (2006) demonstrated that *Marinobacter* sp. dominated biofilms developed from oil fields produced water flow-through systems with nitrate and deemed to play an important role in nitrate treatment to control souring (Kumaraswamy *et al*., 2010). This study demonstrated that *Marinobacter* sp. (Isolate SA005) significantly reduced the weight loss of the stainless steel coupons with supplementation of sodium nitrate as compared to the weight losses observed with the addition of ammonium nitrate. This evidence suggests that these microorganisms might contribute to heterotrophic nitrate reduction suppressing sulfidogenesis (Kumaraswamy *et al*., 2010). *Halomonas*, *Marinobacter* and *Pseudomonas* sp. are identified as putative heterotrophic nitrate reducers and may be associated with nitrate reduction coupled to sulphide oxidation (Kumaraswamy *et al*., 2010).

Although previous microbiological studies have suggested that SRB play a key role in MIC (Graves and Sullivan, 1996; Pope and Pope, 1998; Drysdale *et al*., 1999), in this study, reduced levels of the SRB population were detected in the corrosion product samples obtained from corroded stainless and mild steel coupons in the previous chapters using MPN. It should be noted that Zhu *et al*. (2003) reported that SRB was not detected in biofilm samples obtained from a corroded metal coupon in liquid samples in a gas pipeline. Previous studies suggest that SRB need not be present in abundance in the microbial communities responsible for MIC (Zhu *et al*., 2003; Jan-Roblero *et al*., 2004). Additionally, Maruthamuthu *et al*. (2005) suggested that the water-soluble corrosion inhibitor addition reduced pH values and suppressed SRB proliferation.

MIC is not directly related to the total biofilm biomass but is to the specific combinations of microbes in that biofilm (Jack *et al*., 1992). The proposed mechanism of bacterial inhibition of corrosion could be that bacterial biofilms might prevent chloride attack on steel surfaces, the changes of the localized environmental chemistry by bacterial metabolites and oxygen depletion at metal surfaces by respiring cells. The current results prove that the isolated aerobic microorganisms do play a role in the corrosion process in both stainless steel and mild steel coupons. In addition, it can be noted that certain isolated bacterial species do possess the potential of reducing corrosion on metal coupons however, many non-culturables could not be isolated using the standard methods used in this chapter. Therefore, a molecular approach needs to be followed to isolate other potential bacterial isolates that may play a significant role in the corrosion process.

CHAPTER FIVE
MICROBIAL COMMUNITY PROFILING OF MICROORGANISMS PRESENT IN BIOFILMS ON CORRODED METAL COUPONS

5.1 INTRODUCTION

Traditional culture techniques have yielded valuable information about microbial interactions within the environment however, only a small fraction of microorganisms can be cultured (Wen and Hong-Bo, 2011). It is generally accepted that the vast majority of microorganisms detectable by indirect methods in marine and other natural samples, including those collected from much less extreme environments, cannot be assessed by traditional methods that rely on laboratory cultivation (Harmsen *et al*., 1997). In addition, spatial heterogeneity and aggregation may compromise culture enumeration (Amann *et al*., 1992). Numerous studies have been performed to determine the composition of the communities present in biofilms in various environments (Macdonald and Brozel, 2000). These technical hurdles have been partly overcome by the development of culture-independent molecular methods that often lie in the universal distribution and sequence conservation of the rRNA molecules (Harmsen *et al*., 1997). In many studies conventional cultivation techniques were used in conjunction with molecular techniques to determine the spectrum of microorganisms present in biofilms, since it was repeatedly shown that less than 1 % of the bacterial population from oligotrophic environments could be cultured (Macdonald and Brozel, 2000). The culturing approach has yielded a skewed picture of biofilm communities and has been described as "the great plate count anomaly" by Staley and Konopka (1985).

Applications of molecular biology to the identification of microorganisms in the environment have revolutionized microbial identification (Harmsen *et al*., 1997; Macdonald and Brozel, 2000). The genetic material (DNA, RNA) inside bacteria are unique for all individual species (Wen and Hong-Bo, 2011). In particular, the 16S rRNA molecule is perhaps the most widely used nucleic acid target for detection and identification of previously undetected organisms (Harmsen *et al*., 1997; Macdonald and Brozel, 2000). The 16S rRNA sequencing and analysis method is an important way to infer phylogeny of bacteria and their evolution, which gives a comparative analysis of the homology of 16S rRNA sequences of different bacteria (Wen and Hong-Bo, 2011). At the heart of the revolution was the development of labelled probes for the *in situ* identification of

microorganisms by hybridization to ribosomal RNA (Harmsen *et al*., 1997; Macdonald and Brozel, 2000). By using the sequences of base pairs in the 16S rRNA, probes can be designed to target individual groups, generas or strains of bacteria (Wen and Hong-Bo, 2011). The 16S rRNA-targeted Fluorescence *In Situ* Hybridization (FISH) techniques have been successfully applied to the direct identification of microbial cells from the highest to the lowest phylogenetic levels and their accurate enumeration in genetically diverse natural assemblages (Harmsen *et al*., 1997). The technique based on binding of a fluorescent-labeled oligonucleotide probe to 16S rRNA is generally used to estimate the numbers of ribosome-rich bacteria in samples (Icgen and Harrison, 2006).

Fluorescently labelled rRNA-targeted oligonucleotides are used to specifically differentiate members of microbial communities. The specificity of the probes ranges from the phylotype to the kingdom, depending on the targeted region of the rRNA (Hesham and Alamri, 2012). FISH is considered useful for many applications in all fields of microbiology, as this technique allows simultaneous visualization, identification, enumeration and localization of individual microbial cells (Tabatabaei *et al*., 2009). The dyed cells can then be counted with epifluorescence microscopy (Rogers *et al*., 2007). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content (Hesham and Alamri, 2012). The rapid and sensitive FISH technique with multiple oligonucleotide probes provides information on the qualitative and quantitative composition of microbial communities, providing a general picture of microbial diversity and determining the phylogenetic position of uncultured microorganisms (Rogers *et al*., 2007; Hesham and Alamri, 2012).

Nucleic acid hybridization and comparative sequence analyses are increasingly being applied to studies of environmental microbiology. These applications have been fostered by developments in both rapid sequencing techniques and comparative sequence analyses. Notably, comparative sequencing of large rRNAs (16S-like and 23S-like) has been served to cast both microbial classification and the design of nucleic acid hybridization probes within a phylogenetic framework (Amann *et al*., 1992). These probes may be labelled with fluorescent groups, radioactive groups or antigens for immunological detection, and have been used with great success in a variety of ecosystems (Macdonald and Brozel, 2000). Oligonucleotide probes complementary to specific regions of rRNAs have been fabricated for the identification of both individual and phylogenetically coherent assemblages of microorganisms. Such probes are increasingly used to quantify natural

microbial populations and to identify new isolates, serving to help alleviate reliance upon pure-culture isolation and phenotypic identification (Amann *et al*., 1992).

All these approaches have advantages as well as limitations to their use in natural ecosystems (Macdonald and Brozel, 2000). The reliability and reproducibility of these methods are affected by technical factors like efficiency of DNA extraction, PCR biases and selection of clones (Tabatabaei *et al*., 2009). Some methodological problems include cell permeability, target site accessibility, target site specificity and sensitivity. Cell permeability is achieved by the fixation of cells with denaturants such as alcohols, or cross-linking reagents such as formaldehyde or paraformaldehyde. A further problem, especially in natural systems, is the signal strength of the fluorescently labelled probes. In oligotrophic environments, where the cells in biofilms often have a slow growth rate, and as a result a low ribosome count due to low nutrient concentration, the attainable signal per cell is weak, necessitating signal amplification (Macdonald and Brozel, 2000).

Until recently, little has been known about corrosive bacteria because of their difficulty to grow; thus the application of culture-dependant methods has been shown to be difficult to apply and has led to incorrect results and conclusions. Therefore the application of culture-independant techniques for detection and identification of microorganisms involved in biocorrosion is necessary (Kawai *et al*., 2002; Kjellerup *et al*., 2009). Denaturing gradient gel electrophoresis (DGGE) has become one of the most frequently used methods for molecular fingerprinting (Sanchez *et al*., 2007). PCR of 16S ribosomal DNA fragments followed by DGGE is particularly effective in analyzing the microbial community structure of non-culturable environmental samples (Muyzer *et al*., 1993; Long *et al*., 2008; Xiuheng *et al*., 2009). PCR-DGGE of 16S rRNA obtains profiles of microbial communities that can be used to identify temporal or spatial differences in community structure or to monitor shifts in the structure that occurs in response to environmental perturbations (Park and Crowley, 2005; Wen and Hong-Bo, 2011). This method has been introduced into molecular ecology to determine the genetic diversity of natural microbial communities and to identify the phylogenetic affiliation of community members by DNA sequence analysis of amplified fragments after they have been excised (Kawai *et al*., 2002; Wen and Hong-Bo, 2011).

The DGGE method can provide direct sequence information useful for the assessment of the different phylogenetic groups present (Tabatabaei *et al*., 2009). DGGE is a commonly used technique for investigating the diversity of bacteria and archae in the natural environment. DNA is

extracted directly from environmental samples and used as a template for PCR amplification of 16S rRNA genes originating from resident prokaryotic populations. DGGE primer pairs include a GC clamp at the 5' end of the forward primer; this facilitates the migration of PCR products of similar size but varying sequence, to different locations within a polyacrylamide gel containing a denaturing gradient of urea and formamide. A profile of prokaryotic diversity is generated, where each band represents the 16S rRNA gene of a different species in the original sample. In order to identify phylogenetic affiliation, DGGE bands are routinely excised from the gel, re-amplified with the original primers, and sequenced directly from the PCR product (O'Sullivan *et al*., 2008).

The detection of sulphate reducers and sulphate-reducing activity in sediments, wastewater treatment plants, and fouling biofilms is of great practical and scientific relevance. Conventional microbial techniques based on selective culturing are of limited usefulness for quantification and characterization of environmental populations, as it is now well recognised that most strains do not grow *in vitro*, either because cultivation media poorly resemble natural growth conditions or because different strains of microorganisms are interdependent (Brock, 1987; Wagner *et al*., 1993). Techniques based on the analysis of bacterial DNA and RNA may complement the conventional microbiological approach and nowadays are routinely used to determine the presence and distribution of individual bacterial species, including SRB, in complex communities such as those in bacterial biofilms (Raskin *et al*., 1996). Studies relating to community structure and community function are scarce, partially because of difficulties in monitoring microbial activities. This complicates the interpretation of community function analysis because extrapolation of community behaviour to that of individual cells is impossible without knowledge of their microenvironment (Santegoeds *et al*., 1998).

The results obtained in Chapters 2 and 3 display the microbial population involved in the corrosion process of both stainless and mild steels. However, the bacterial species isolated were limited due to the presence of many non-culturables in the environments tested. Chapter 4 focused on the isolation of culturables and their potential in corrosion activation or inhibition however, to obtain a more clear observation of the microbial population involved in biofilm formation, corrosion enhancement and inhibition, molecular techniques are used. This chapter reports on the study of the composition of biofilms present on the surface of mild steel coupons by fluorescent *in situ* hybridization using fluorescently labelled 16S rRNA-targeted oligonucleotide probes. Other molecular techniques used

were DGGE analysis of PCR-amplified 16S ribosomal DNA (rDNA) fragments to determine the complexity of the microbial community in the biofilm and to monitor its behaviour over time.

5.2 MATERIALS AND METHODS

5.2.1 EXPERIMENTAL SYSTEM SET-UP

Metal coupons were extracted from the stimulated seawater/sediment and loam soil systems as described in Sections 2.2.2 and 3.2.2 in Chapter 2 and Chapter 3 respectively.

5.2.2 MICROBIAL POPULATION STUDIES OF CORRODED COUPONS USING FLUORESCENT *IN SITU* HYBRIDIZATION

5.2.2.1 SAMPLE EXTRACTION AND FIXATION OF BIOFILM CELLS

The microbial population was extracted from the biofilms of corroded coupons as described by Macdonald and Brozel (2000). Biofilms were scraped off from the surface of corroded mild steel coupons using a sterile surgical blade (carbon steel surgical blade sterilized by GAMMA radiation). The biofilm scrapings were diluted in 10 ml 1 x PBS. A 2 ml volume of the cell suspension (planktonic and attached) was centrifuged for 5 mins (7000 rpm) and the supernatant discarded. The pellet was resuspended in 100 µl 0.5 % glucose and 0.5 % yeast extract, and incubated for 2 hrs at 30 °C (Macdonald and Brozel, 2000). Following the incubation, 20 µl of chloramphenicol (5 µg/µl) was added and further incubated for 1 hr. The pellet was washed twice in 1 x PBS and cells were fixed by adding 0.1 volume 37 % formaldehyde (Merck) and incubated for 24 hrs at 4 °C. Cells were harvested and resuspended in 50 µl 1 x PBS, after which 50 µl 96 % ethanol was added and the cells were stored at -20 °C.

5.2.2.2 HYBRIDIZATION OF FIXED BIOFILM CELLS

The fixed biofilm cells were hybridized according to the methods described by Ito *et al*. (2002) and McLeod *et al*. (2002). A 3 µl volume of fixed cells in suspension was spotted onto gelatin-coated slides (Appendix 1) and air-dried for 2 hrs at 37 °C. Slides were washed consecutively in 50 %, 70

% and 90 % (v/v) ethanol for 3 mins each. A pre-warmed mixture of 300 µl of hybridization buffer (1 volume of probe working solution, 50 ng DNA μl^{-1}, in 9 volumes of hybridization buffer) was added to the coated slides and incubated at 46 °C for 2 hrs in a sealed moisture chamber. Subsequently, a stringent washing step was performed at 48 °C for 1 hr in 50 ml of pre-warmed washing solution. Washing buffer was removed by rinsing the slides in double-distilled water and the slides were allowed to air-dry. The slides were stored at 4 °C in the dark.

The following 16S rRNA-targeted oligonucleotide probes were used: EUB 338, NON 338 as a negative control, and SRB 385. The general probe (EUB338) and the SRB385 probe were used for targeting bacteria, the families Desulfovibrionaceae and Desulfobulbaceae within the Deltaproteobacteria respectively. All probe sequences, their specificities and hybridization conditions are given in Table 5.1. Probes EUB 338 and SRB 385 were synthesized and labeled with Cy 3.5 and HEX at the 5' end respectively. Non-specific binding to the samples was tested by the fluorescein isothiocyanate-labeled Nonsense probe NON 338 (Inqaba Biotech, South Africa).

All *in situ* hybridizations were performed in 8 µl of hybridization buffer, 0.01 % sodium dodecyl sulfate and variable formamide concentrations as described in Table 5.1 with 1 µl of probe solution at 46 °C for 2 hrs in an equilibrated sealed moisture chamber. The final probe concentration was approximately 5 ng μl^{-1}. This was followed by a stringent washing step at 48 °C for 20 mins in 50 ml of prewarmed washing solution (variable NaCl concentrations as described in Table 5.1, 20 mM Tris-HCl, pH 7.2, 0.01 % SDS). Washing buffer was removed by rinsing the slides with double-distilled water and the slides were allowed to air dry.

Table 5.1: 16S rRNA-targeted oligonucleotide probes used in this study (Ito *et al*., 2002)

Probe	**Specificity**	**Sequence of probe (5'-3')**	**Target site[a]**	**FA[b] Conc (%)**	**NaCl[c] (mM)**
EUB 338	Domain *Bacteria*	GCTGCCTCCCGTAGGAGT	338-355	20	0.166
NON 338	None (negative control)	ACTCCTACGGGAGGCAGC	338-355	0	0.900
SRB 385	SRB of the δ subdivision of the proteobacteria plus several gram-positive bacteria (e.g. *Clostridium* spp.)	CGGCGTCGCTGCGTCAGG	385-402	30	0.071

[a] 16SrRNA position according to *E.coli* numbering.
[b] Formamide concentration in the hybridization buffer.
[c] sodium chloride concentration in the washing buffer.

5.2.2.3 FLUORESCENCE *IN SITU* HYBRIDIZATION AND ANALYSES OF PROBE-HYBRIDIZED CELLS

The slides were mounted with an anti-fading mountant, Mowiol (Appendix 1) and were examined using a fluorescent microscope. All positively probe-hybridized cells were directly counted and an average total cell count per area (μm^2) was reported (Ito *et al*., 2002). All image processing and analyses were performed with the standard imaging iTEM software (Universal TEM Imaging Platform, Soft Imaging System, based on analySIS Five).

5.2.3 MICROBIAL POPULATION STUDIES OF CORRODED COUPONS USING DENATURING GRADIENT GEL ELECTROPHORESIS

5.2.3.1 DNA EXTRACTION AND PCR AMPLICATION OF 16S rDNA GENES

A sample of biofilm (0.1 g) was scraped directly off the surface of the corroded mild steel coupon using a sterile surgical blade. DNA was extracted using the MoBio Soil DNA Extraction kit according to manufacturer's instructions. The conserved region of 16 rDNA was amplified by PCR (Zhu *et al*., 2003). The reaction mixture for PCR amplification contained 1 µl each of forward and reverse primers (50 pmol μL^{-1} each, Inqaba Biotech), 25 µl ReadyMix Taq PCR Reaction Mix with $MgCl_2$ (2x), (Fermentas Life Sciences) and 1 µl of template DNA. One microlitre of Bovine Serum Albumin (10 mg/ml) was added to the reaction mixture at the expense of 1 µl of nuclease-free water, when PCR-amplifying DNA from biofilm samples (Kjeldsen *et al*., 2007). The primers used in the reaction were forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998). The composition of the PCR Master Mix (2x) was 0.05 u/ µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). PCR was conducted using the ThermoHybaid PCR Express Thermal Cycler (Ashford, Middlesex) under the following cycling parameters: 30 cycles of initial denaturation at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 mins, followed by the final elongation step at 72 °C for 5 mins (1 cycle) (Sambrook *et al*., 1989). The PCR products were evaluated electrophoretically on a 1 % (wt/vol) agarose gel at 80 V for 90 mins in 1x Tris-Acetate – EDTA running buffer, stained with ethidium bromide and visualized with

the Chemi Genius^2 BIO Imaging System and Gene Snap software (Syngene, UK). Product size was determined using Generuler DNA ladder mix (Fermentas).

5.2.3.2 DENATURING GRADIENT GEL ELECTROPHOREIS ANALYSIS

Following confirmation of amplification of the 16S rRNA gene, a further round of amplification was carried out with a primer set of 357F with a 25 bp GC clamp at the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3') and the universal primer 518R (5'-ATT ACC GCG GCT GCT GG-3'). The cycling parameters were as follows: initial denaturation at 95 °C for 5 mins, followed by 10 cycles: 94 °C for 30 secs, 55 °C for 30 secs and 72 °C for 1 min followed by 26 cycles of PCR: 92 °C for 30 secs, 52 °C for 30 secs and 72 °C for 1 min with a final extension step at 72 °C for 10 mins (O' Sullivan *et al*., 2008). DGGE was performed using the Dcode^{TM} Universal Mutation Detection System (Bio-Rad) with the following reagents and conditions: 1x TAE, 8 % thick gels and a denaturant gradient containing 30 to 60 % urea-formamide at 60 °C, at 200 V constantly for 4 hrs. A DGGE marker was constructed using the isolates studied in Chapter 4, namely, *B. megaterium* (LMA1), *Marinobacter* sp. (SA003), *Pseudomonas* sp. (LMS21), *B. idriensis* (LS007), *B. megaterium* (NL003), *Lysinibacillus* sp. (LMS2), *B. thuringiensis* (LA002), *Bacillus* sp. (LMA2), *B. aerophilus* (CL002), *B. pumilus* (CS001) and *Halomonas* sp. (SA004). Two microlitres of DNA from each isolate were mixed together and amplified with 357F-GC and 518R primers as described previously. The DGGE gels were stained with ethidium bromide and visualized with the Chemi Genius^2 BIO Imaging System and Gene Snap software (Syngene, UK).

5.2.3.3 DNA SEQUENCING AND PHYLOGENETIC ANALYSIS OF DGGE BANDS

Selected DGGE bands were carefully excised and subjected to PCR amplification according to Ye *et al*. (2009). The excised bands were eluted in 100 µl of millipore water and incubated overnight at 4 °C. Thereafter, the bands were spun down at 8000 rpm for 5 mins. The upper layer was extracted and used at the template. PCR amplification of the excised bands was carried out with primers 357F (without a GC clamp) and 518R. The cycling parameters were as follows: initial denaturation at 95 °C for 5 mins, followed by 30 cycles: 94 °C for 30 secs, 58 °C for 1 min and 72 °C for 1 min followed by a final extension step at 72 °C for 5 mins. Amplified products were sequenced with the

518R primer by Inqaba Biotech (South Africa). The BLASTN search tool (Altschul *et al*., 1990; Altschul *et al*., 1997) was used to determine sequence homology and the most similar sequences in the GenBank database. The most similar sequences obtained by database searches were used to construct multiple alignments using the Mega4 (Tamura *et al*., 2007). The phylogenetic relationships were determined using the neighbour-joining bootstrap method using the Mega 4 software (Tamura *et al*., 2007).

5.2.3.4 PHYLOGENETIC ANALYSIS OF DGGE BANDING PROFILES

Following Muyzer *et al*. (1993) each detected band was referred to as a phylotype. The phylotype profiles of the samples from the two different environments were compared by Sorenson's index, $C_s = 2j / (a + b)$, a pairwise similarity coefficient (Murray *et al*., 1996), where j is the number of common DGGE bands, a is the number of DGGE bands in the autoclaved control, and b is the number of DGGE bands in the experimental samples. A C_s value of 0 indicates that the samples are completely different, and a C_s value of 1 indicates that the samples are identical.

5.3 RESULTS

5.3.1 FLUORESCENT *IN SITU* HYBRIDIZATION ANALYSES (FISH)

The relative abundance of members of the domain *Bacteria* detected with probes EUB338 and NON338, and of SRB detected with family- or genus-specific probes on the biofilm scrapings off corroded mild steel coupons were determined by FISH. The total number of cells that hybridized to the probes per area (μm^2) was determined. This technique was used to determine the relative abundance of some major bacterial taxonomic groups. Since FISH labels only bacteria with a certain amount of rRNA, only active cells or cells that have been recently active will be enumerated. Biofilm cells were scraped from the coupons into PBS and allowed to increase in size using glucose and yeast extract while preventing cell division by addition of chloramphenicol followed by hybridization.

Figures 5.1 and 5.2 present a series of composite images of biofilm scrapings of mild steel coupons immersed in the nitrate-treated systems in the stimulated seawater/sediment system after 4, 12 and 20 weeks of incubation (the images of the autoclaved control and the non-autoclaved samples can

be found in Appendix VII). These images display the successional development of the heterogenous biofilm structure and SRB populations *in situ*. The probe SRB385-hybridized cells were present in the forms of single scattered cells as seen in Figures 5.1 and 5.2. The degree of fluorescence is known to be proportional to the ribosome count, which in turn is proportional to the growth rate. From the images obtained, it is clear that EUB338 targeting bacteria were more prominent with more cells showing fluorescence than those hybridized with the SRB385 probe indicating decreased population growth of SRB.

Table 5.2 displays the total cell counts (average) ± standard deviation per area (μm^2) for probes in the stimulated seawater/sediment system based on the FISH images below. Most counts of positively-hybridized cells in the seawater/sediment system for each probe decreased over the 20 week study period. A decrease in SRB-hybridized cells (3.50±1.72 x 11285.78 μm^2 and 7.20±1.99 x 11498.75 μm^2 for the nitrate-treated samples) and the EUB-hybridized cells (6.50±3.03 x 11091.15 μm^2 and 11.40±5.21 x 10839.80 μm^2 for the non-autoclaved and ammonium nitrate-treated sample respectively) were observed. The number of SRB385-hybridized cells gradually decreased with time from 8.20±3.08 x 11170.04 μm^2 at 12 weeks to 4.64±1.69 x 9877.45 μm^2 at 20 weeks in the non-autoclaved sample. A similar trend is observed in all other samples.

After four weeks of incubation, the number of cell counts for both EUB338 and SRB385 probes were 52.40±25.43 x 11246.86 μm^2 and 52.80±39.15 x 11349.87 μm^2 respectively in the autoclaved control (Table 5.2). At the initial stage, it seems that the number of cells counted were similar for both probes. After four weeks of incubation there were higher counts in the non-autoclaved sample for the EUB338 probe (36.80±16.54 x 10765.02 μm^2) than for the SRB385 probe (10.69±4.03 x 10475.85 μm^2) (Table 5.2). There were higher counts of SRB-hybridized cells (29.20±13.47 x 10603.01 μm^2) than the EUB-hybridized cells (13±7.30 x 11386.76 μm^2) in the sample supplemented with sodium nitrate (Figure 5.1). However, after four weeks of incubation, the counts for the positive EUB- hybridized cells and SRB-hybridized cells were lower than that detected in the autoclaved control for both probes and for the EUB probe in the non-autoclaved sample. In the sample supplemented with ammonium nitrate, lower counts were observed for both EUB and SRB-hybridized cells (11±6.33 x 11416.78 μm^2 and 6.83±11092.26 μm^2 respectively) as compared to all samples.

The weight loss measurements (Figure 2.2 in Section 2.3.3 of Chapter 2) of the mild steel coupons displayed a weight loss of 3.545 mg/g in the non-autoclaved sample than that observed in the autoclaved control (2.58 mg/g). This higher weight loss measurement is indicative that the higher counts of hybridized cells demonstrates that the microbial communities present in the heterogenous biofilm play a role in the weight loss of the coupons. There were also higher counts of hybridized cells detected in the samples supplemented with sodium nitrate than ammonium nitrate; the weight loss measurements were 9.78 mg/g and 11.49 mg/g for sodium nitrate and ammonium nitrate-treated samples respectively.

After 20 weeks of incubation the number of SRB-hybridized cells were much lower (4.64±1.69 x 9877.45 μm^2) than that observed in EUB-hybridized cells (6.50± 3.03 x 11091.15 μm^2) in the non-autoclaved sample and to those values in the autoclaved control (8.33±2.61 x 11554.20 μm^2 and 18.70±9.63 x 10838.41 μm^2 for SRB and EUB-hybridized cells respectively). This was also observed with lower weight loss measurement (7.09 mg/g) in the non-autoclaved sample than the 10.845 mg/g weight loss in the autoclaved control. Lower counts were observed for the SRB-probe (3.50±1.72 x 11285.78 μm^2) than the EUB-probe (18.40±7.41 x 11517.70 μm^2) in the sodium nitrate supplemented sample which displayed a weight loss of 22.49 mg/g. A similar trend was observed in the samples supplemented with ammonium nitrate (Figure 5.2), where there were higher counts for EUB-hybridized cells (11.50±6.33 x 11416.78 μm^2) than for SRB-hybridized cells (6.83±6.32 x 11092.26 μm^2) and displaying a weight loss measurement of 20.97 mg/g. However, after 12 weeks of incubation the total cell counts for both probes increased and after 20 weeks of incubation the cell counts decreased for all samples.

Table 5.2: *In Situ* Hybridization image analysis of the mild steel sample from the seawater sediment system displaying the total cells per area (μm^2) at different sampling times

WEEKS	EUB338	NON338	SRB385
AUTOCLAVED			
4	52.40±25.43 x11246.86	15.30±7.87 x 10719.26	52.80±39.15 x 11349.87
12	26±10.30 x 11344.68	23.42±11.80 x 11183.15	12.45±6.47 x 11130
20	18.70±9.63 x 10838.41	5.33±4.13 x 11712.82	8.33±2.61 x 11554.20
NON-AUTOCLAVED			
4	36.80±16.54 x 10765.02	16.77±9.92 x 10364.18	10.69±4.03 x 10475.85
12	20.10±8.08 x 11036.23	5.90±3.38 x 10965.02	8.20±3.08 x 11170.04
20	6.50±3.03 x 11091.15	8±3.65 x 11456.61	4.64±1.69 x 9877.45
SODIUM NITRATE			
4	13±7.30 x 11386.76	37.91±11 x 10427.51	29.20±13.47 x 10603.01
12	11.80±6.09 x 10998.23	17.70±7.76 x 11483.09	5.50±1.78 x 10955.61
20	18.40±7.41 x 11517.70	3±1.83 x 11491.45	3.50±1.72 x 11285.78
AMMONIUM NITRATE			
4	11.50±6.33 x 11416.78	6±4.06 x 10875.99	6.83±6.32 x 11092.26
12	23.82±11.24 x 11065.40	7.60±2.80 x 11398.10	14.20±8.66 x 11148.76
20	11.40±5.21 x 10839.80	5.80±4.18 x 11309.14	7.20±1.99 x 11498.75

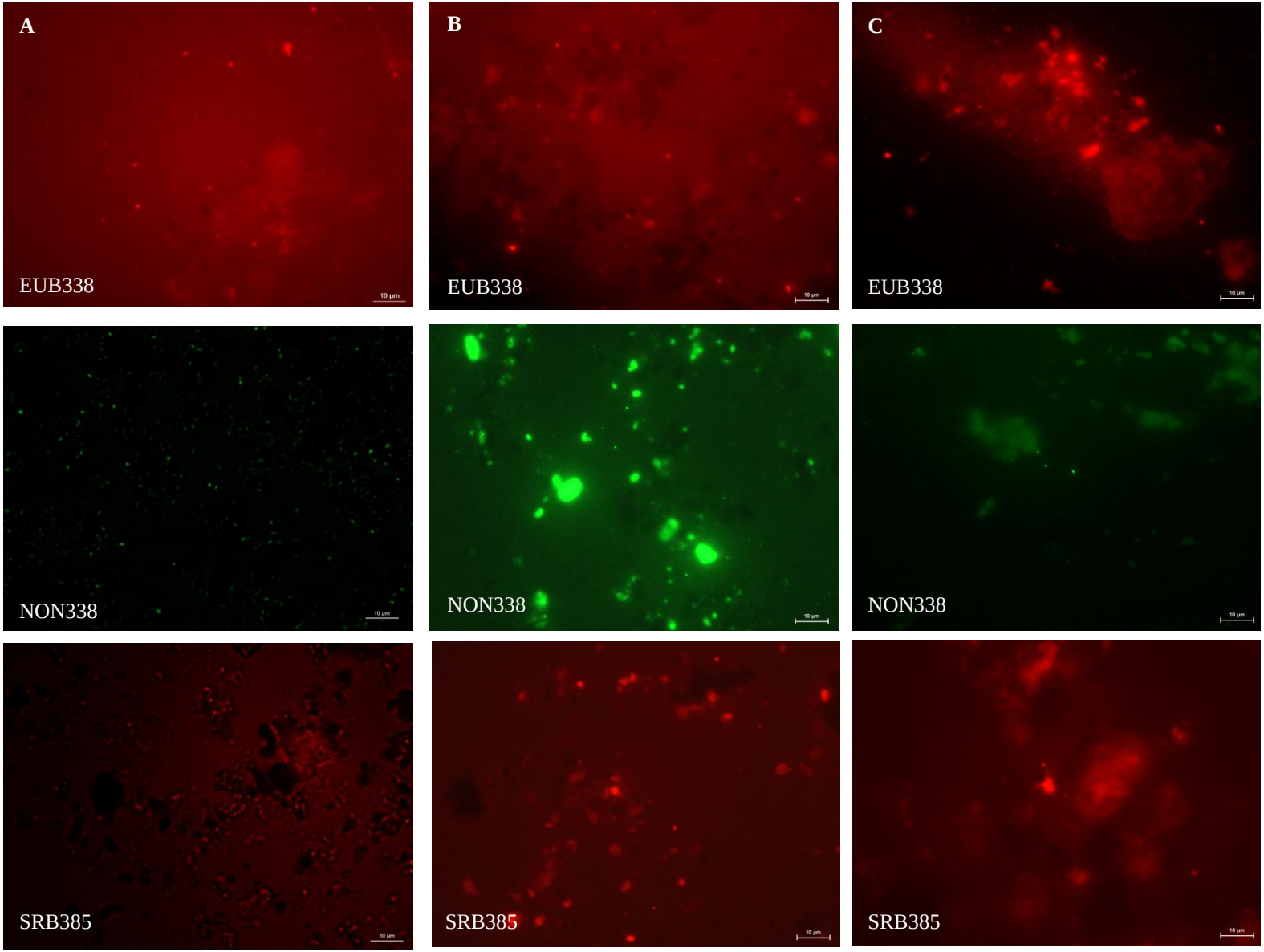

Figure 5.1: Composite images of biofilm scrapings from the mild steel sample supplemented with sodium nitrate from the seawater/sediment system with EUB338, NON338 and SRB385-hybridized probes after 4(A), 12(B) and 20(C) weeks of incubation

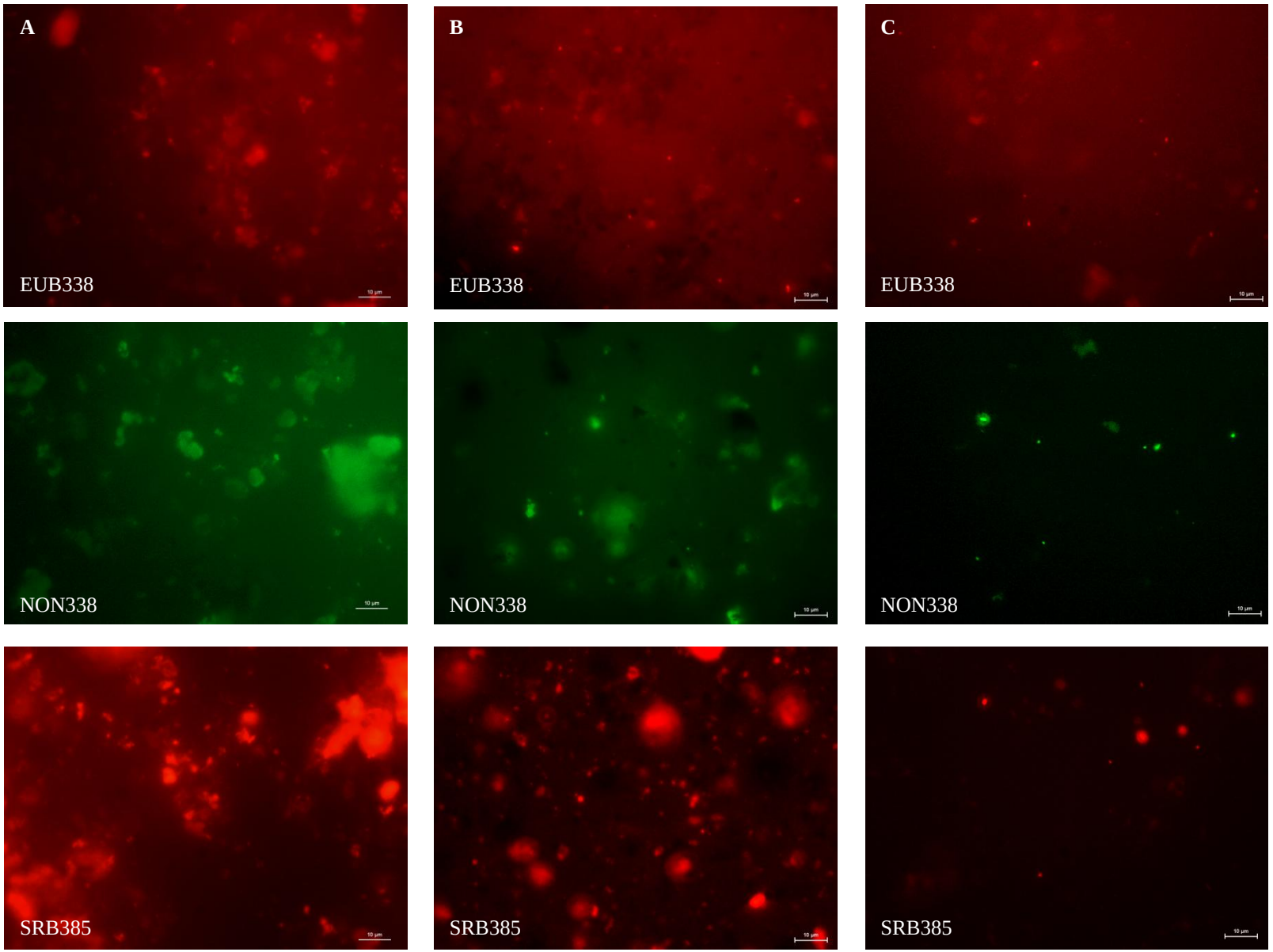

Figure 5.2 Composite images of biofilm scrapings from the mild steel sample supplemented with ammonium nitrate from the seawater/sediment system with EUB338, NON338 and SRB385-hybridized probes after 4(A), 12(B) and 20(C) weeks of incubation

A similar trend was observed in the stimulated loam soil system as that observed in Table 5.2. Figures 5.3 and 5.4 present a series of composite images of biofilm scrapings of mild steel coupons immersed in the nitrate-treated systems in the loam soil system after 4, 12 and 20 weeks of incubation. The images of the autoclaved control and the non-autoclaved samples can be found in Appendix VII. Table 5.3 displays the total cell counts (average) ± standard deviation per area (μm^2) for probes in the stimulated loam soil system. After four weeks of incubation the number of SRB-hybridized cells was lower than EUB-hybridized cells (11.09±4.93 x11367.38 μm^2 and 6.42±2.81 x 11500.89 μm^2 respectively) in the non-autoclaved sample, but was higher than that observed in the autoclaved control (2.70±1.83 x 10783.87 μm^2) for SRB-hybridized cells (Table 5.3). The non-autoclaved sample however, displayed a lower weight loss measurement of 6.8 mg/g of the mild steel coupons as compared to 7.075 mg/g in the autoclaved control (as seen in Figure 3.2 in Section 3.3.3 of Chapter 3).

The counts for both probes increased after 12 weeks of incubation (38.70±10.06 x 1529.31 μm^2 and 12.50±4.95 x 1505.21 μm^2 for EUB- and SRB-hybridized cells respectively) however, after 20 weeks of incubation, the number of hybridized cells (6.91±4.25 x 11268.61 μm^2 and 4.10±1.66 x 11187.10 μm^2 for EUB- and SRB-hybridized cells respectively) decreased in the non-autoclaved loam soil sample. This is consistent with the weight loss measurements observed, with a higher weight loss of 11.83 mg/g observed after 12 weeks with a decrease in weight loss of 2.465 mg/g after 20 weeks of incubation. The number of SRB-hybridized cells was low throughout the study, but was observed to be much lower after 20 weeks of incubation in all samples. After 20 weeks of incubation the number of EUB-hybridized cells (25±14.03 x 11179.78 μm^2) was higher than the SRB-hybridized cells (5.33±2.64 x 10916.83 μm^2) in the autoclaved control, with higher cell counts than the non-autoclaved sample (6.91±4.25 x 11268.61 μm^2 and 4.10±1.66 x 11187.10 μm^2 for EUB- and SRB-hybridized cells respectively). These counts coincide with the weight loss measurement with higher weight loss (2.9 mg/g) observed in the autoclaved control and in the non-autoclaved sample (2.465 mg/g).

The number of EUB-hybridized cells (6.40±1.67 x 10776.65 μm^2) was lower than that observed with the SRB-probe (8±3.27 x 11436.68 μm^2) after four weeks of incubation in the sample supplemented with sodium nitrate (Figure 5.3). After 12 weeks of incubation the number of cells hybridized with EUB338 probe increased (24.73±11.23 x 1525.89 μm^2) and decreased after 20 weeks of incubation (3±2.36 x 11252.87 μm^2). This was observed with an increase in weight loss

measurements (19.03 mg/g and 26.735 mg/g after 12 and 20 weeks of incubation respectively) as shown in Figure 3.2.

After four weeks of incubation there were higher counts for SRB-hybridized cells (6.60±2.46 x 11239.35 μm^2) than the EUB-hybridized cells (3.90±1.20 x 11177.22 μm^2) in the sample supplemented with ammonium nitrate which displayed a weight loss of 5.565 mg/g (Figure 5.4). The counts for both probes increased after 12 weeks of incubation in the sample supplemented with ammonium nitrate with 16.353 mg/g weight loss and thereafter decreased after 20 weeks of incubation with a weight loss measurement of 4.36 mg/g (Table 5.3).

The number of EUB-hybridized cells was higher (25±14.03 x 11179.78 μm^2) in the autoclaved loam soil sample after 20 weeks of incubation as compared to that observed in the seawater/sediment system (18.70±9.63 x 10838.41 μm^2). In the non-autoclaved sample, a similar trend was observed in the number of EUB- and SRB-hybridized cells in both stimulated systems. In the nitrate treated systems however, an increase in the number of both EUB- and SRB-hybridized cells was observed in the stimulated seawater/sediment system than that observed in the loam soil system after 20 weeks of incubation.

Table 5.3: *In Situ* Hybridization image analysis of the mild steel samples from loam soil displaying the total cells per area (μm^2)

WEEKS	EUB338	NON338	SRB385
AUTOCLAVED			
4	11.10±6.03 x 10756.52	3.60±1.26 x 10915.21	2.70±1.83 x 10783.87
12	46.70±12.65 x 1526.30	15±4.99 x 1526.95	17.85±7.88 x 1523.02
20	25±14.03 x 11179.78	21.40±12.41 x 11128.18	5.33±2.64 x 10916.83
NON-AUTOCLAVED			
4	11.09±4.93 x11367.38	4.79±3.45 x 15657.69	6.42±2.81 x 11500.89
12	38.70±10.06 x 1529.31	11.40±3.98 x 1529.08	12.50±4.95 x 1505.21
20	6.91±4.25 x 11268.61	3.10±1.29 x 11181.57	4.10±1.66 x 11187.10
SODIUM NITRATE			
4	6.40±1.67 x 10776.65	3.90±1.37 x 11285.44	8±3.27 x 11436.68
12	24.73±11.23 x 1525.89	9±6.85 x 1529.32	8.30±3.77 x 1517.90
20	3±2.36 x 11252.87	1.83±1.17 x 11006.88	2.50±1.08 x 11134.55
AMMONIUM NITRATE			
4	3.90±1.20 x 11177.22	3±1.33 x 11473	6.60±2.46 x 11239.35
12	27.5±4.38 x 1520.78	5.70±4.50 x 1508.85	16±4.83 x 1517.66
20	9±4.82 x 11354	3.50±3.21 x 11179.79	4.50±2.51 x 10990.38

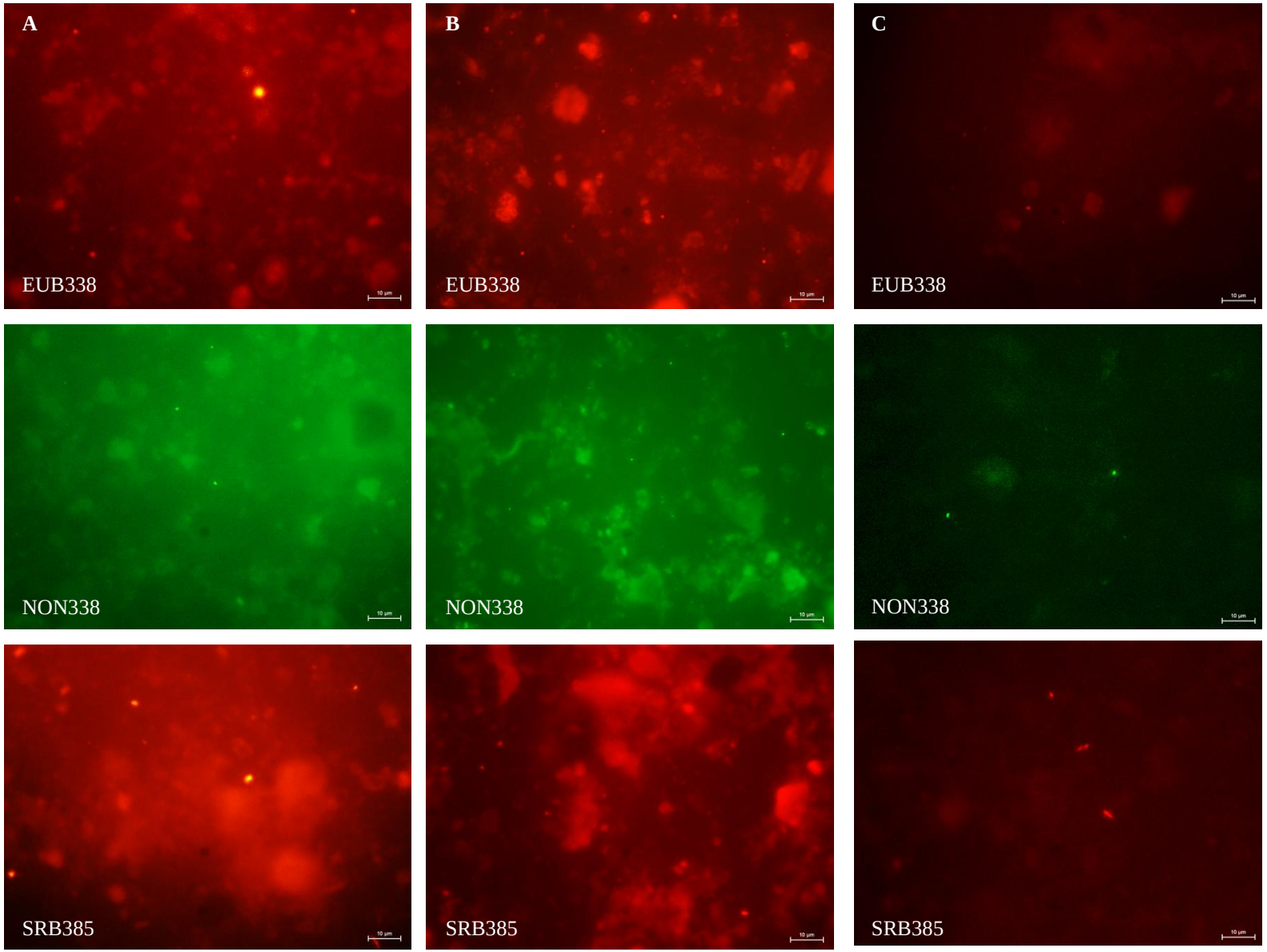

Figure 5.3: Composite images of biofilm scrapings from the mild steel sample supplemented with sodium nitrate from loam soil with EUB338, NON338 and SRB385-hybridized probes after 4(A), 12(B) and 20(C) weeks of incubation

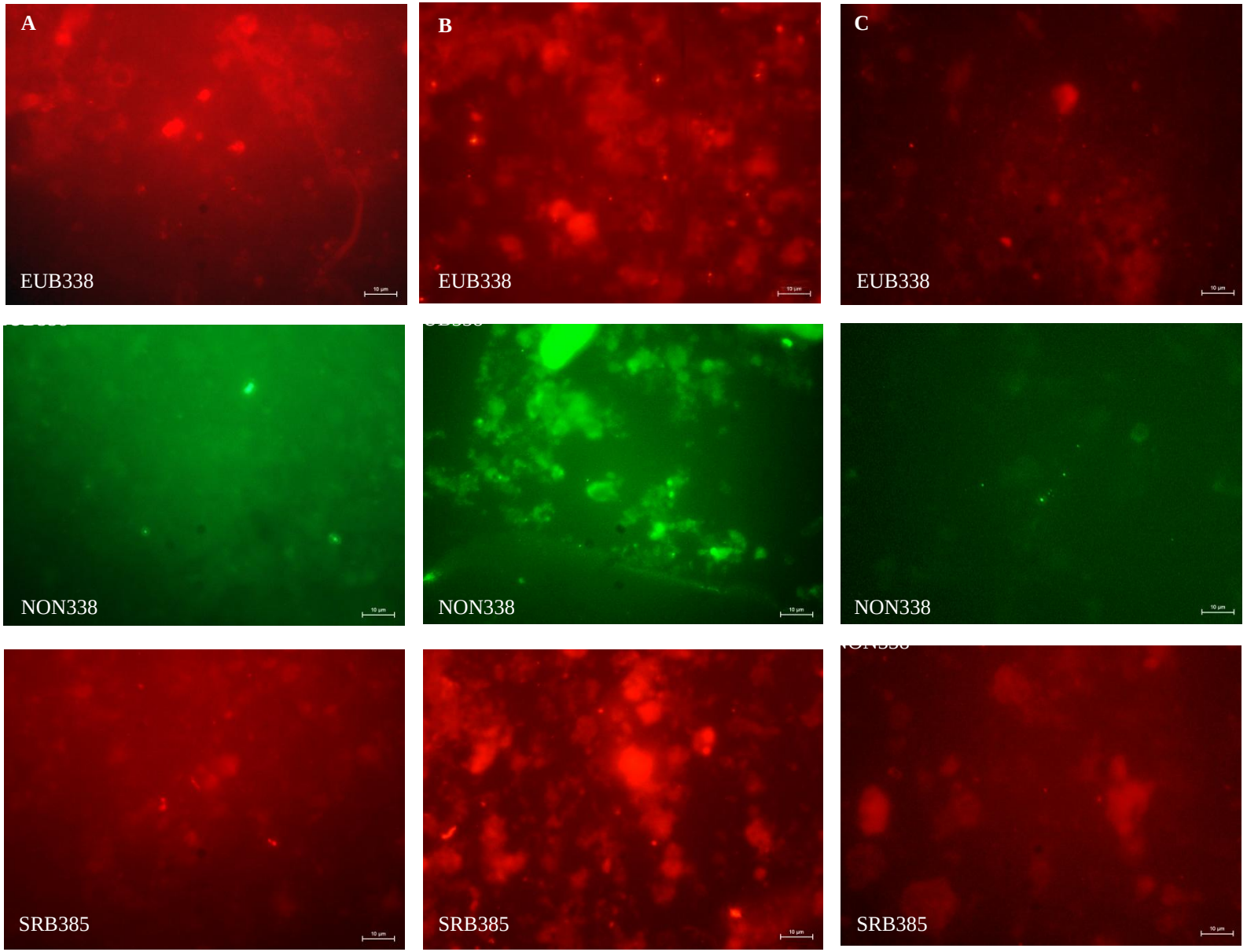

Figure 5.4: Composite images of biofilm scrapings from the mild steel sample supplemented with ammonium nitrate from loam soil with EUB338, NON338 and SRB385-hybridized probes after 4(A), 12(B) and 20(C) weeks of incubation

5.3.2 DGGE ANALYSIS OF PCR-AMPLIFIED rDNA FRAGMENTS AND PHYLOGENETIC AFFILIATION OF DOMINANT BACTERIA

The biofilm was scraped off the surface of corroded mild steel coupons from both the stimulated seawater/sediment and loam soil systems at different sampling times. DNA was extracted from these biofilm scrapings and amplified with the primer pair 63F and 1387R. Figure 5.5 shows PCR amplification of the 16S rDNA gene of biofilm samples after 20 weeks of incubation in both the stimulated seawater/sediment and loam soil systems. The product size after PCR was estimated to be 1324 bp. After confirmation of 16S rDNA amplications the DNA was then amplified with the DGGE primer pair 357F-GC and 518R, with a resulting amplicon of 161 bp after 20 weeks of incubation, as seen in Figure 5.6. PCR amplification of the 16S rDNA gene and amplification with the DGGE primers of the samples from both stimulated systems after 4, 8, 12 and 16 weeks of incubation are shown in Appendix VIII.

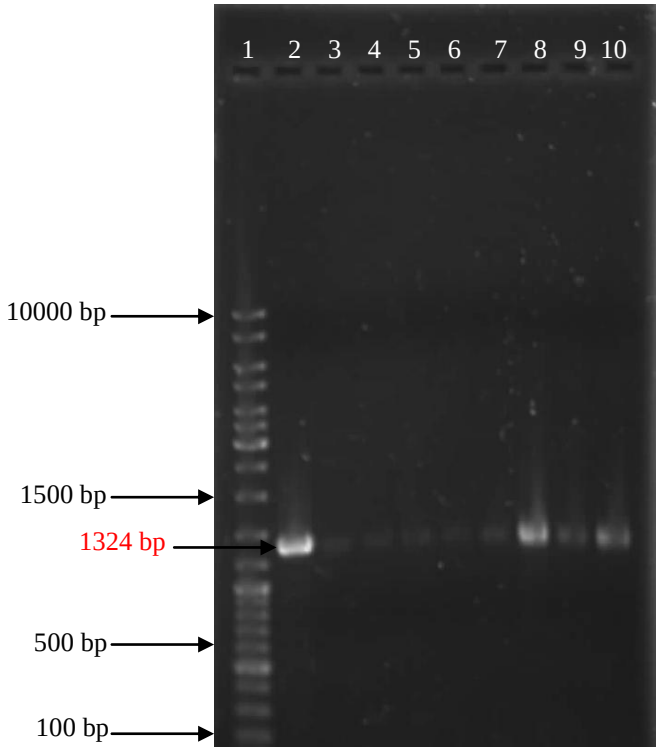

Figure 5.5: PCR amplication of the 16S rDNA gene of biofilm samples after 20 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: molecular weight marker (Fermentas); 2: positive control (cDNA isolated from previous study); 3: autoclaved control in the seawater/sediment system; 4: non-autoclaved sample in the seawater/sediment system; 5: sodium nitrate in the seawater/sediment system; 6: ammonium nitrate in the seawater/sediment system; 7: autoclaved control in the loam soil; 8: non-autoclaved sample in the loam soil; 9: sodium nitrate in the loam soil and 10: ammonium nitrate in the loam soil)

Amplicons of 16S rDNA fragments with a GC clamp, obtained after extraction of nucleic acids from the biofilm samples that were scraped off the mild steel coupons immersed in both the seawater/sediment and loam soil systems, were analyzed by DGGE. The resulting pattern of bands was visualized by gel electrophoresis and ethidium bromide staining. Figure 5.6 shows PCR amplification with the chosen DGGE primers of the biofilm samples after 20 weeks of incubation. Samples were also analyzed after 4, 8, 12 and 16 weeks of incubation in both stimulated systems (Appendix VIII).

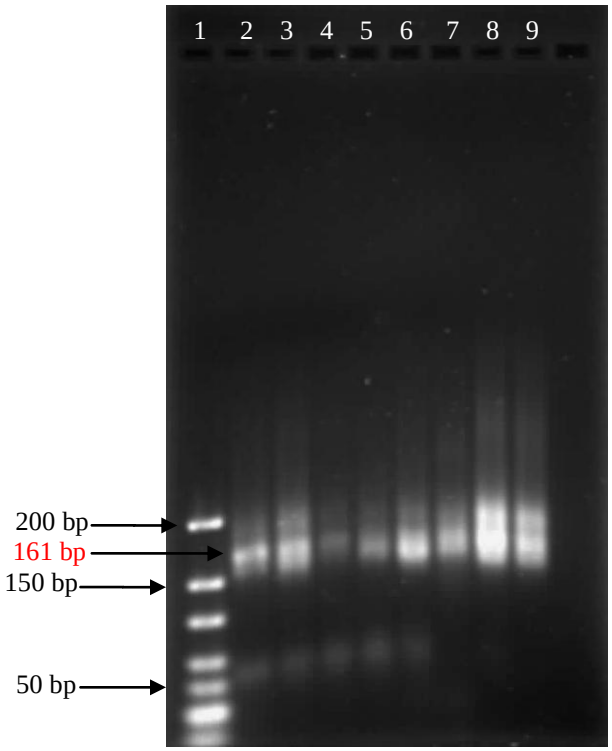

Figure 5.6: PCR amplication with DGGE primers of biofilm samples after 20 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: molecular weight marker (Fermentas); 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil)

DGGE analysis of amplicons, obtained with the primer pair 357F-GC and 518R, generated multiple bands from the DNA extracts from coupons extracted from both stimulated systems at sampling times of 4, 8, 12, 16 and 20 weeks. The number of individual bands obtained in a DGGE analysis is related to the number of bacterial species in the sample. Since each band can be considered as an operational taxonomy unit (OTU), the DGGE profiles showed that dominant

microbial species were 16, 13, 12, 11 and 16 OTUs derived from the 4, 8, 12, 16 and 20 week samples respectively (Figures 5.7 to 5.11). This indicates a diverse microbial community present on the surface of the mild steel coupons in both stimulated systems. Microbial community structure was much more complex at four and 20 weeks. Dominant bands were shared among the samples at different sampling times showing a large degree of commonality between the samples despite the difference in both stimulated systems.

Several fragments detected in the biofilm samples from mild steel coupons extracted from both the seawater/sediment and loam soil systems were excised and reamplified by PCR. Partial 16S rDNA sequences of approximately 161 bases were obtained from the DNA originating from each dominant band. The sequences obtained were submitted to a BLAST search to retrieve the corresponding phylogenetic relatives. The sequence data confirmed that the bacterial diversity revealed phylotypes affiliated to seven distinct major phylogenetic groups (Alpha-, Beta-, Gamma-, Deltaproteobacteria, Actinobacteria, Lactobacillales and Firmicutes). Neighbour-Joining phylogenetic trees were constructed for the excised DGGE bands from distances calculated using the Maximum Composite Likelihood method. Eukaryotic *Amorphotheca resinae* and *Penicillium* sp. were chosen as outgroups. The similarities and species identified with the phylogenetic analysis for the bands excised after 4, 8, 12, 16 and 20 weeks of incubation are given in Tables 5.4 to 5.8. Some bands were consistent in the other DGGE gels after 8, 12, 16 and 20 weeks incubation when compared to the gel after four weeks of incubation and therefore were not sequenced but assumed to be the same as those that appeared in Figure 5.7.

The increased number of bands indicated a diverse microbial community in both stimulated systems after four weeks of incubation (Figure 5.7). The number of bands was similar in the non-autoclaved and the nitrate-treated samples in both systems indicating the presence of *Bacillus* sp. and *Pseudomonas* sp. being more dominant. This indicated that during the early stages of the study there was no major shift in population diversity observed in response to the nitrate treatments. No nitrate-utilizing bacteria or SRB were detected during the early stages of the experiment with the addition of nitrates.

Table 5.4 shows the 47 bands that were excised and sequenced (Figure 5.7). Many of the bands showed affiliation to the Alpha-, Gamma- and Delta-proteobacteria, Firmicutes, Clostridia and Cyanobacteria. A total of eight excised bands were sequenced but could not be affiliated to a putative group. Two organisms affiliated to the Clostridia group were detected from lane 9 (No.

8) (loam soil supplemented with ammonium nitrate) and lane 3 (non-autoclaved seawater/sediment sample). A phylogenetic tree illustrating samples from the non-autoclaved seawater/sediment sample (Figure 5.8) showed that isolated *Clostridium thermopalmarium* (No.33) and *Cohaesibacter* sp. (No. 35) formed a support with the other isolated DGGE bands, forming a clade with very good support (99 % bootstrap). Within this clade, the isolated *Bacillus* sp. (No. 47), *Lysinibacillus* sp. (No. 42), and *Halomonas* sp. (No. 49) (SA004, JN106414 as described in Chapter 4 and constructed as a molecular marker) formed a clade with 28 % bootstrap support.

An affiliation to Flavobacterium was detected from lane 5 (seawater/sediment sample supplemented with ammonium nitrate). Cyanobacteria were detected in the loam soil supplemented with ammonium nitrate (Lane 9, No.2). The sequence obtained from the loam soil sample supplemented with sodium nitrate (Lane 8, No.10) showed 100 % similarity to the anaerobic, Gram-negative, halophilic, SRB, *Desulfohalobium retbaense*. In loam soil samples with additional sodium nitrate (Figure 5.9), isolated *Thiomonas* sp. (DGGE band 33, 20 week sample) and uncultured bacterium clone (49 % bootstrap) formed a clade with *Desulfohalobium retbaense* (65 % bootstrap). The majority of the isolated DGGE bands (3 % bootstrap) formed a clade with isolated *Terribacillus saccharophilus* (13 % bootstrap). This clustered clade formed another clade with *Bacillus* sp. (DGGE band 12, four week sample) with 27 % bootstrap support. This clade formed a further clade with isolated *Acinetobacter calcoaceticus* with good support (94 % bootstrap).

Psuedomonas fluorescens (Nos. 25 and 16) was an isolate found in both the ammonium nitrate-treated sample in the seawater/sediment system and the autoclaved loam soil sample after four weeks of incubation. *Methylobacterium* sp. was the only dominant band found in the ammonium nitrate-treated loam soil sample. *Bacillus* sp. was determined to be a dominant species in the seawater/sediment samples, except in the sodium nitrate-treated sample however, this bacterial isolate was detected in the sodium nitrate-treated loam soil sample.

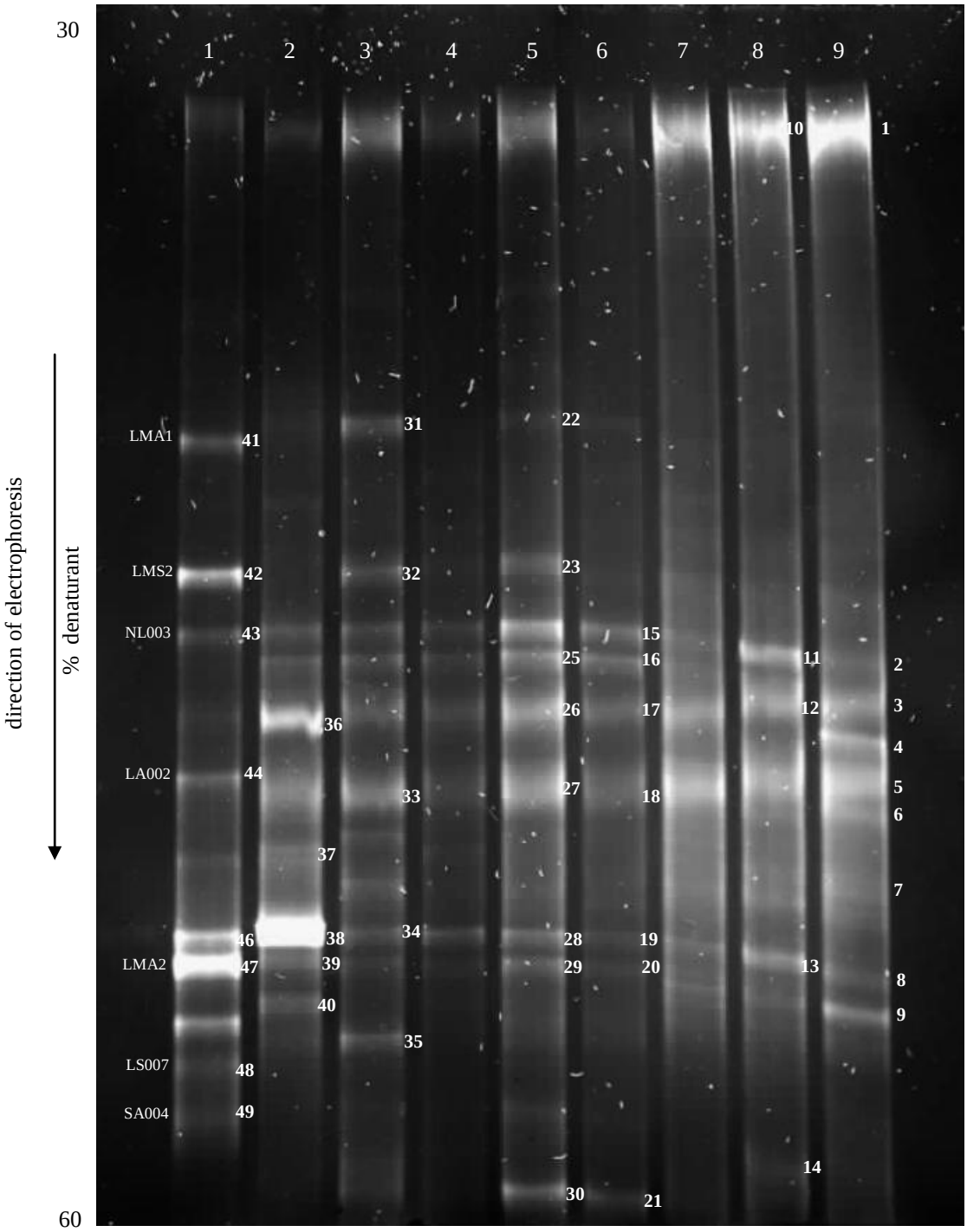

Figure 5.7: DGGE profile of biofilm samples after 4 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: DGGE markers; 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil). The numbers indicate gel portions that were excised for re-amplification and sequencing.

Table 5.4: Partial 16S rDNA sequence similarity of the excised bands on DGGE profiles of 4 week samples

No	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION	No	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION	No	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION
1	*Methylobacterium* sp. EX135 (GQ342553)	86 %	*α-proteobacteria*	17	*Bacillus* sp. ITRI62 (FJ013334)	75 %	*Firmicutes*	34	*Bacillus* sp. H3B1 (GU212628)	83 %	*Firmicutes*
2	*Acaryochloris marina* MBIC11017 (CP000828)	92 %	*Cyanobacteria*	18	*Mesorhizobium tianshanense* strain NBRC 102499 (AB681836)	91 %	*α-proteobacteria*	35	*Cohaesibacter* sp. DQHS-21 (GQ200200)	97 %	*α-proteobacteria*
3	Uncultured *Acidobacterium* group bacterium clone N11.111WL (AF431406)	81 %	-	19	*Mesorhizobium* sp. NL123 (AB636289)	93 %	*α-proteobacteria*	36	Soil bacterium 13G-15 (EU839355)	100 %	-
4	Uncultured bacterium clone BFM13CONT3RFd06 (JF696109)	72 %	-	20	*Mesorhizobium plurifarium* strain ORS 3356 (DQ859036)	89 %	*α-proteobacteria*	37	Soil bacterium 13G-15 (EU839355)	100 %	-
5	*Methylobacterium* sp. iRV5 (AY358015)	73 %	*α-proteobacteria*	21	*Mesorhizobium tianshanense* strain NBRC 102499 (AB681836)	100 %	*α-proteobacteria*	38	Soil bacterium 13G-16 (EU839356)	100 %	-
6	Uncultured *Raoultella* sp. isolate DGGE gel band N04 (GU272216)	74 %	*γ-proteobacteria*	22	*Methylocystis minimus* (L20844)	75 %	*α-proteobacteria*	39	*Bacillus* sp. Hs73 (JF803866)	99 %	*Firmicutes*
7	Uncultured *Raoultella* sp. isolate DGGE gel band N04 (GU272216)	74 %	*γ-proteobacteria*	23	*Chryseobacterium* sp. CH5 (EU598808)	79 %	*Flavobacteriia*	40	*Bacillus* sp. JCM 10530 (AB689744)	99 %	*Firmicutes*
8	Uncultured *Clostridium* sp. isolate DGGE gel band 14 (HM164430)	76 %	*Clostridia*	25	*Pseudomonas fluorescens* (EF408245)	95 %	*γ-proteobacteria*	41	*Bacillus megaterium* strain NBIGP (JN004170)	100 %	*Firmicutes*
9	Uncultured soil bacterium clone CRS5557T-2 (GU366000)	91 %	-	26	*Brevibacillus* sp. phR (JN837488)	76 %	*Firmicutes*	42	*Lysinibacillus* sp. MD001 (JN160728)	99 %	*Firmicutes*
10	*Desulfohalobium retbaense* DSM 5692 (CP001734)	100 %	*δ-proteobacteria*	27	*Roseobacter* sp. TKW (DQ479380)	93 %	*α-proteobacteria*	43	*Bacillus megaterium* strain AIMST (HQ694028)	91 %	*Firmicutes*
11	*Acinetobacter calcoaceticus* strain PUCM1008 (FJ816055)	81 %	*γ-proteobacteria*	28	*Bacillus* sp. H3B1 (GU212628)	85 %	*Firmicutes*	44	*Bacillus thuringiensis* strain AIMST (JN036714)	99 %	*Firmicutes*
12	*Bacillus* sp. bubt21 (JF904525)	78 %	*Firmicutes*	29	*Pseudomonas pseudoalcaligenes* strain AP03 (JF938593)	88 %	*γ-proteobacteria*	46	*Bacillus megaterium* strain EIF18 (HM480345)	100 %	*Firmicutes*
13	*Terribacillus saccharophilus* strain NA24 (JN585721)	92 %	*Firmicutes*	30	Uncultured soil bacterium clone GO0VNXF07H53RD (JF393434)	100 %	-	47	*Bacillus* sp. AKB-2008-4285C (AM988959)	97 %	*Firmicutes*
14	*Bacillus vireti* strain YNB17 (JN700200)	79 %	*Firmicutes*	31	Uncultured bacterium P15-B11 (FN296117)	100 %	-	48	*Bacillus idriensis* strain HCD-7002 (FN813443)	99 %	*Firmicutes*
15	*Acinetobacter* sp. Wr1B05 (JF488005)	97 %	*γ-proteobacteria*	32	*Lysinibacillus fusiformis* strain JTP-23 (GU172172)	81 %	*Firmicutes*	49	*Halomonas* sp. SA004 (JN106414)	94 %	*γ-proteobacteria*
16	*Pseudomonas fluorescens* (EF408245)	89 %	*γ-proteobacteria*	33	*Clostridium thermopalmarium* strain NMY5 (EF639852)	92 %	*Clostridia*				

-: unaffiliated group

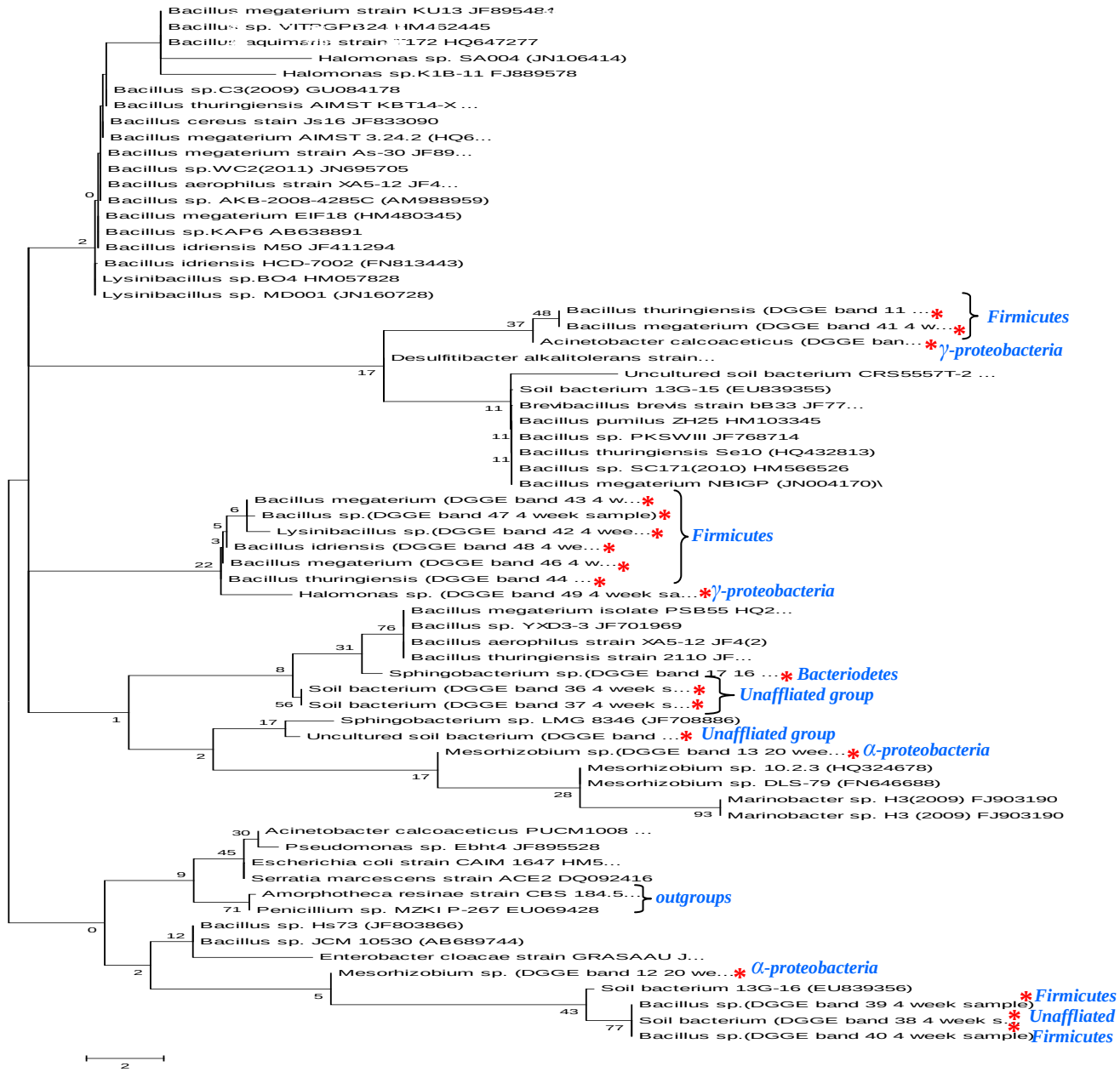

Figure 5.8: The evolutionary history was inferred using the Neighbor-Joining method for the non-autoclaved seawater/sediment sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 116 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

Figure 5.9: The evolutionary history was inferred using the Neighbor-Joining method for the sodium nitrate-treated loam soil sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 97 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

The DGGE banding patterns observed at eight weeks (Figure 5.10) indicated limited bacterial diversity within the microbial population. A greater microbial diversity was observed in the stimulated seawater/sediment system as compared to the loam soil system. Banding profiles are somewhat similar in all samples at 12 weeks (Figure 5.13), although it does reveal limited microbial diversity. Although these results indicate limited banding patterns, suggesting limited microbial populations, it is possible that the nitrate treatment concentration was too low to cause any observable change in the population.

At eight weeks (Table 5.5) many of the excised bands were sequenced and revealed affiliations to the Alpha- and Gamma-proteobacteria, Lactobacillales and Actinobacteria. One of the excised bands could not be affiliated to a putative group after sequencing. The band excised from the seawater/sediment sample supplemented with ammonium nitrate (Lane 4) as seen in Figure 5.10, revealed a 95 % sequence similarity to an aerobic, Gram-negative, nitrate-reducing marine bacterium, *Nitratireductor kimnyeongensis*. In Figure 5.11 (seawater/sediment system with additional sodium nitrate samples) uncultured *Shewanella* sp. formed a clade with the *E. coli* strain and *S. marcescens* strain (15 % bootstrap). *B. thuringiensis* and *B. megaterium* (DGGE bands 44 and 46 respectively) formed a clade (55 % bootstrap) with *B. idriensis* (DGGE band 48) forming a further clade with only 4 % bootstrap. *Nitratireductor kimnyeongensis* and *Halomonas* sp. formed a clade (6 % bootstrap) with two isolated bands of *B. megaterium* (DGGE bands 41 and 43). In Figure 5.12 (seawater/sediment system with additional ammonium nitrate samples) *Brevibacillus* sp. (DGGE band 26, four week sample) formed a clade (40 % bootstrap) with other isolated DGGE bands (20 % bootstrap).

Streptococcus thermophilus (No. 6) is a dominant isolate found after eight weeks of incubation in the sodium nitrate-treated loam soil sample. Soil bacterium (No. 11) detected in the seawater/sediment sample supplemented with ammonium nitrate is the same as that detected in the autoclaved seawater/sediment sample (Figure 5.7, No. 37) after four weeks of incubation. *Pseudomonas* sp. (No. 10) was detected in the ammonium nitrate-treated seawater/sediment sample after four and eight weeks of incubation.

Table 5.5: Partial 16S rDNA sequence similarity of the excised bands on DGGE profiles of 8 week samples

BAND	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION
3	*Agrobacterium tumefaciens* strain IGS8 (JN680248)	84 %	*α-proteobacteria*
4	*Agrobacterium tumefaciens* strain IGS43 (JN680214)	80 %	*α-proteobacteria*
5	*Lactobacillus sakei* (DQ336385)	90 %	*Lactobacillales*
6	*Streptococcus thermophilus* clone GM-2 (DQ001071)	96 %	*Lactobacillales*
7	*Rhodopseudomonas* sp. JA558 (FN813495)	98 %	*α-proteobacteria*
8	Uncultured *Bifidobacterium* sp. (FN178330)	96 %	*Actinobacteria*
10	*Pseudomonas* sp. IGS26 (JN680199)	100 %	*γ-proteobacteria*
11	Soil bacterium 13G-15 (EU839355)	100 %	-
13	*Nitratireductor kimnyeongensis* strain NBRC 106418 (AB682431)	95 %	*α-proteobacteria*

-: unaffiliated group

The excised bands from the 12 week samples showed affiliation to the Firmicutes, Lactobacillales, Actinobacter, Gamma- and Betaproteobacteria (Table 5.6). In Figure 5.13 the band excised in Lane 5 (seawater/sediment sample supplemented with ammonium nitrate) showed 75 % sequence similarity to *Marinobacter* sp. In the non-autoclaved loam soil sample, the excised band revealed a 88 % sequence similarity to *Micromonospora* sp. In the autoclaved loam soil samples (Figure 5.14) isolated DGGE bands belonging to *Acinetobacter* sp., *Rhodopseudomonas* sp., and uncultured *Bifidobacterium* sp. (6 % bootstrap), formed a clade (with no support) with a clade formed with isolated *Acinetobacter calcoaceticus*, *Bradyrhizobium* sp., and uncultured soil bacterium. Isolated *Pseudomonas fluorescens* and *Bacillus* sp. (11 % bootstrap) formed a clade with *Halomonas* sp. KIB-11 (8 % bootstrap). Isolated *Mesorhizobium* sp. (21 % bootstrap) formed a clade (2 % bootstrap) with isolated *B. megaterium* (DGGE bands 46 and 43) and *B. thuringiensis* (19 % bootstrap). In the seawater/sediment sample supplemented with sodium nitrate (Lane 4) there was a 99 % sequence similarity to *Burkholderia cepacia*.

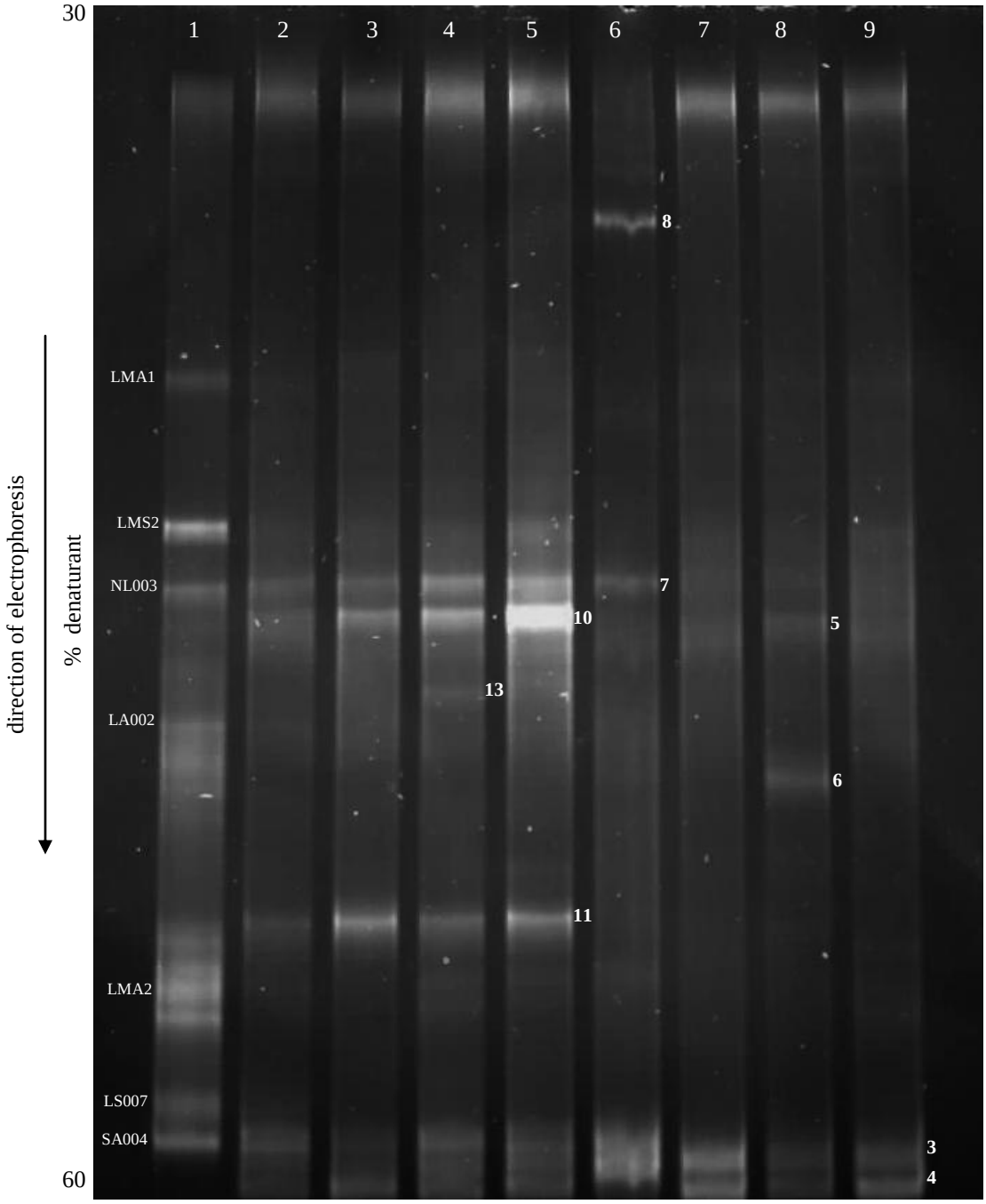

Figure 5.10: DGGE profile of biofilm samples after 8 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: DGGE markers; 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil). The numbers indicate gel portions that were excised for re-amplification and sequencing.

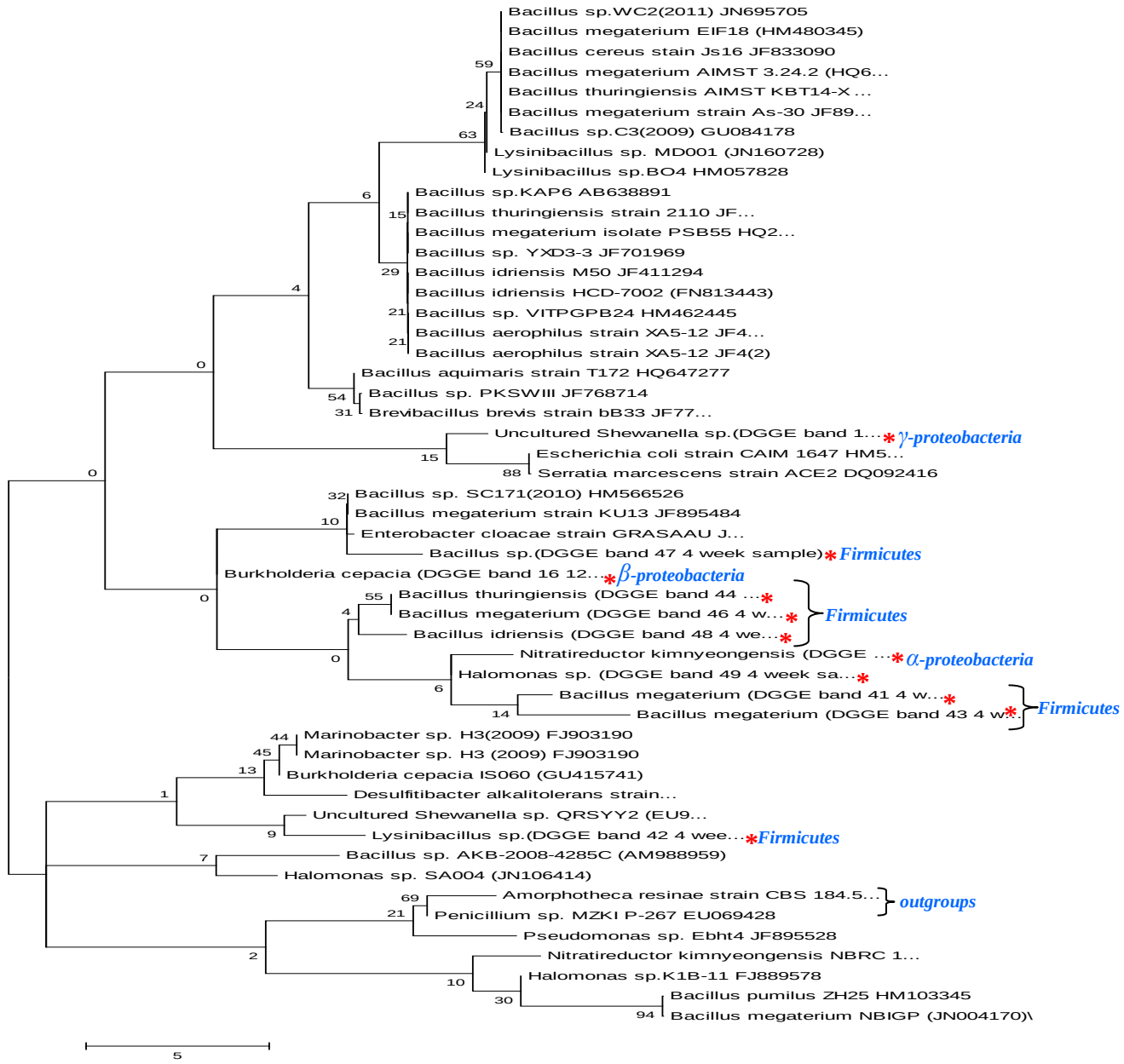

Figure 5.11: The evolutionary history was inferred using the Neighbor-Joining method for the sodium nitrate-treated seawater/sediment sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 110 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

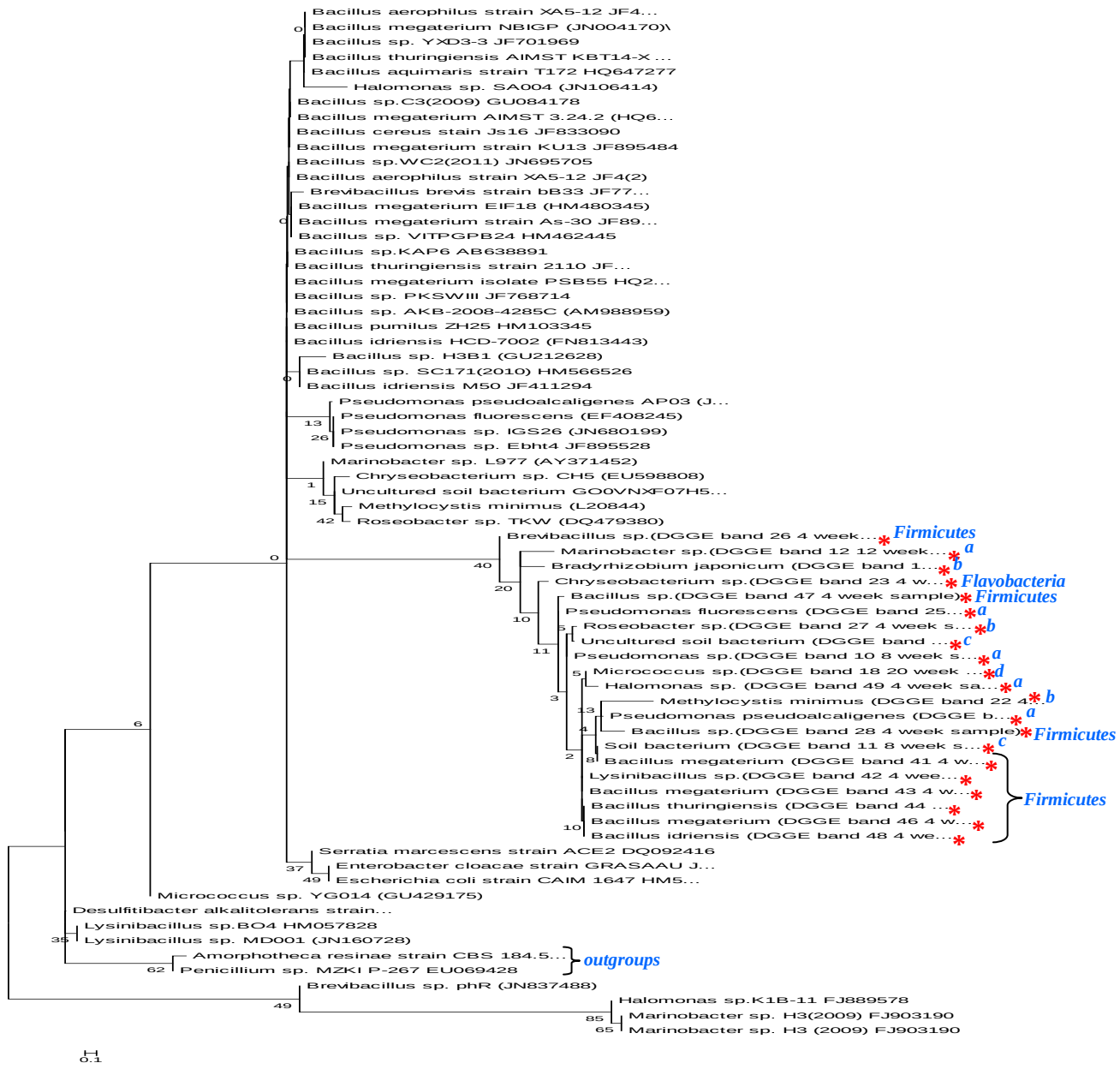

Figure 5.12: The evolutionary history was inferred using the Neighbor-Joining method for the ammonium nitrate-treated seawater/sediment sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 69 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study) [a - *γ-proteobacteria*, b - *α-proteobacteria*, c – unaffiliated group, d – *Actinobacteria*]

Lysinibacillus sp. (No. 10) was detected after 12 weeks of incubation in the autoclaved loam soil sample as well as in the non-autoclaved seawater/sediment sample after four weeks of incubation. *Bacillus* sp. (No. 6) was detected in all samples in both systems which is consistent with that observed in previous DGGE gels (Figures 5.7 and 5.8). *Marinobacter* sp. (No. 12) is a dominant isolate detected in the ammonium nitrate-treated seawater/sediment sample after 12 weeks of incubation.

Table 5.6: Partial 16S rDNA sequence similarity of the excised bands on DGGE profiles of 12 week samples

BAND	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION
6	*Bacillus* sp. OL01 (JN791390)	87 %	*Firmicutes*
7	Uncultured *Trichococcus* sp. clone QRSYY9 (EU919224)	98 %	*Lactobacillales*
9	*Micromonospora* sp. PY09-03 (JN120962)	88 %	*Actinobacteria*
10	*Lysinibacillus* sp. R-46326 (FR775038)	100 %	*Firmicutes*
12	*Marinobacter* sp. L977 (AY371452)	75 %	*γ-proteobacteria*
14	Uncultured *Shewanella* sp. clone QRSYY2 (EU919217)	98 %	*γ-proteobacteria*
16	*Burkholderia cepacia* clone IS060 (GU415741)	99 %	*β-proteobacteria*

An increase in banding patterns was observed in loam soil samples at 16 and 20 weeks (Figures 5.15 and 5.18) as compared to the seawater/sediment samples. At 16 weeks the nitrate-treated samples revealed greater microbial population diversity. An increase in DGGE bands were also observed at 20 weeks in the stimulated seawater/sediment system than that observed during the previous sampling times. This is indicative that time may play a role in conjunction with the nitrate treatments in increasing microbial population diversity.

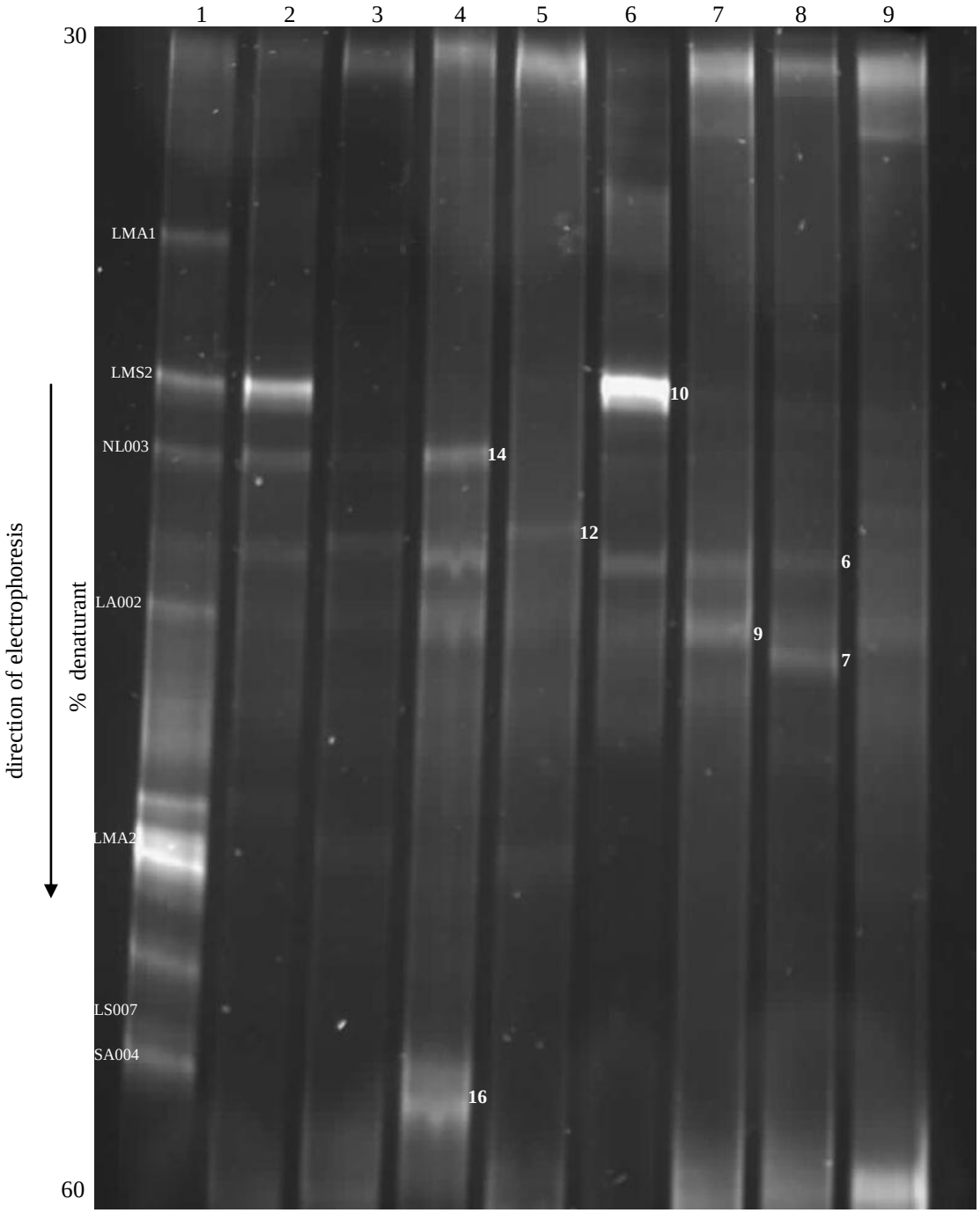

Figure 5.13: DGGE profile of biofilm samples after 12 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: DGGE marker; 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil). The numbers indicate gel portions that were excised for re-amplification and sequencing.

Figure 5.14: The evolutionary history was inferred using the Neighbor-Joining method for the autoclaved loam soil control sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 63 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

The bands excised from the 16 week samples (Table 5.7) revealed affiliation to the Firmicutes, Bacteriodetes, Alpha- and Gammaproteobacteria. Two excised bands could not be affiliated to a putative group. The band excised from Lane 2 (autoclaved control from the seawater/sediment system) revealed a 100 % sequence similarity to *Sphingobacterium* sp. Figure 5.16 illustrated a phylogenetic tree constructed from isolated DGGE bands from the autoclaved control seawater/sediment samples. Isolated DGGE bands revealing *Bacillus* sp. (48 % bootstrap support) formed a clade with isolated *Acinetobacter calcoaceticus* (37 % bootstrap). The majority of the isolated DGGE bands belonging to *Bacillus* sp. and *Lysinibacillus* sp. after four weeks of incubation formed a clade with 22 % bootstrap support with the *Halomonas* sp. (DGGE band 49, 4 week sample).

In the loam soil sample with additional ammonium nitrate, the excised band from Lane 9 revealed a 98 % similarity to *Sphingomonas* sp. In loam soil samples with additional ammonium nitrate (Figure 5.17) isolated *Bacillus* and *Lysinibacillus* sp. formed a weak clade with no support. Isolated *Methylobacterium* sp. (DGGE bands 1 and 5), *B. megaterium*, uncultured soil bacterium and *Clostridium* sp., formed a clade with 7 % bootstrap support. *Sphingomonas* sp. (Nos. 4 and 17) were dominant isolates detected only after 16 weeks of incubation in the ammonium nitrate-treated loam soil sample and the autoclaved seawater/sediment sample respectively. *Bacillus* sp. (Nos. 11 and 7) were detected in the loam soil samples which are consistent with that observed at four and 12 weeks of incubation (Figures 5.7 and 5.9).

Table 5.7: Partial 16S rDNA sequence similarity of the excised bands on DGGE profiles of 16 week samples

BAND	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION
4	*Sphingomonas* sp. RP-B11 (FM997987)	98 %	*α-proteobacteria*
7	*Bacillus* sp. N95(2010) (HQ188670)	96 %	*Firmicutes*
8	Uncultured *Bacilli* bacterium clone QRSYY6 (EU919221)	84 %	-
9	*Acinetobacter calcoaceticus* strain PUCM1008 (FJ816055)	100 %	*γ-proteobacteria*
11	Bacillus sp. H7 (DQ268808)	100 %	*Firmicutes*
12	*Mesorhizobium* sp. 10.2.3 (HQ324678)	99 %	*α-proteobacteria*
17	*Sphingobacterium* sp. LMG 8346 (JF708886)	100 %	*Bacteroidetes*
20	Uncultured soil bacterium clone CRS5557T-2 (GU366000)	98 %	-

-: unaffiliated group

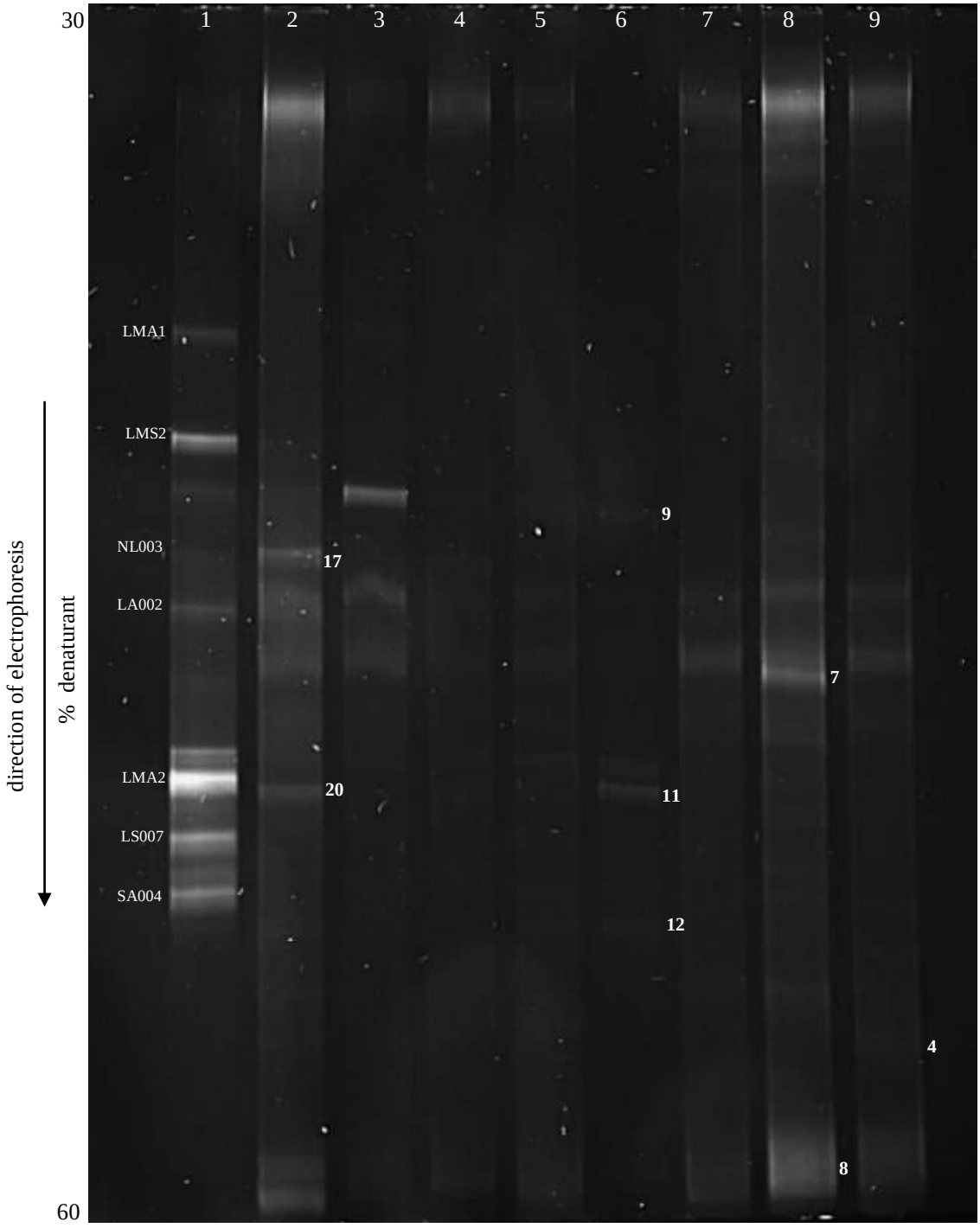

Figure 5.15: DGGE profile of biofilm samples after 16 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: DGGE markers; 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil). The numbers indicate gel portions that were excised for re-amplification and sequencing.

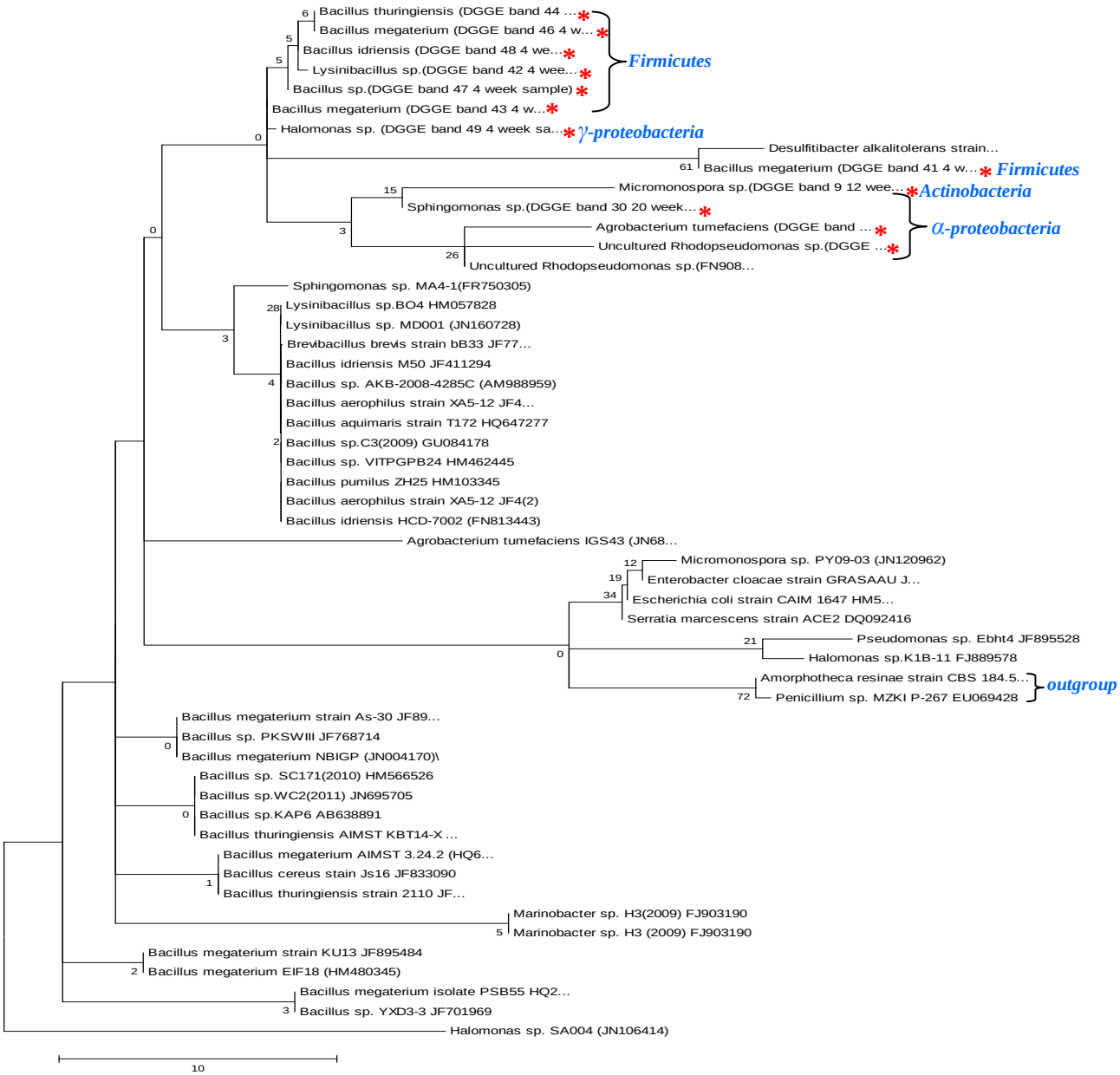

Figure 5.16: The evolutionary history was inferred using the Neighbor-Joining method for the autoclaved seawater/sediment control sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 80 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

Figure 5.17: The evolutionary history was inferred using the Neighbor-Joining method for the ammonium nitrate-treated loam soil sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 12 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study) [a - *γ-proteobacteria*, b - *α-proteobacteria*, c – unaffiliated group, d – *Actinobacteria*]

Two of the excised bands from the 20 week samples (Table 5.8) were sequenced but could not be affiliated to a putative group. The other excised bands were affiliated to the Firmicute, Actinobacteria, Lactobacillales, Alpha-, Beta-, and Gammaproteobacteria. From the seawater/sediment sample supplemented with ammonium nitrate (Lane 5), there was 100 % sequence similarity to *Bradyrhizobium japonicum* (Figure 5.18). In the non-autoclaved loam soil sample (Lane 7), the excised band revealed a 100 % sequence similarity to *Sphingomona* sp. In the non-autoclaved loam soil samples (Figure 5.19), isolated *Micromonospora* sp. (DGGE band 9, 12 week sample) and *Sphingomonas* sp. (DGGE band 30, 20 week sample), (15 % bootstrap) formed a clade (3 % bootstrap) with *Agrobacterium tumefaciens* and uncultured *Rhodopseudomonas* (26 % bootstrap). The clade had no support with other isolated DGGE bands. *Rhodopseudomonas* sp. (No. 19) and *Mesorhizobium* sp. (No. 22) seems to be the dominant isolates after 20 weeks in the autoclaved loam soil sample. *Bradyrhizobium* sp. (Nos. 17 and 23) were only detected after 20 weeks of incubation in the ammonium nitrate-treated seawater/sediment sample and the autoclaved loam soil sample respectively.

Table 5.8: Partial 16S rDNA sequence similarity of the excised bands on DGGE profiles of 20 week samples

BAND	THE CLOSEST SEQUENCES (GENBANK NUMBER)	SIMILARITY	PUTATIVE DIVISION
10	*Acinetobacter calcoaceticus* strain PUCM1008 (FJ816055)	99 %	*γ-proteobacteria*
11	*Bacillus thuringiensis* strain Se10 (HQ432813)	99 %	*Firmicutes*
12	*Mesorhizobium* sp. 10.2.3	93 %	*α-proteobacteria*
13	*Mesorhizobium* sp. DLS-79 (FN646688)	99 %	*α-proteobacteria*
17	*Bradyrhizobium japonicum* USDA (AP012206)	100 %	*α-proteobacteria*
18	*Micrococcus* sp. YG014 (GU429175)	98 %	*Actinobacteria*
19	*Rhodopseudomonas* sp. J5-2 (HM053962)	97 %	*α-proteobacteria*
20	Uncultured soil bacterium clone Bact.wet.ECETB04 (GU375239)	100 %	-
22	*Mesorhizobium* sp. DLS-79 (FN646688)	99 %	*α-proteobacteria*
23	*Bradyrhizobium* sp. 100 (JF905603)	97 %	*α-proteobacteria*
27	*Agrobacterium tumefaciens* strain IGS43 (JN680214)	97 %	*α-proteobacteria*
30	*Sphingomonas* sp. MA4-1 (FR750305)	100 %	*α-proteobacteria*
31	Uncultured *Rhodopseudomonas* sp. (FN908849)	91 %	*α-proteobacteria*
32	*Streptococcus thermophilus* clone GM-2 (DQ001071)	83 %	*Lactobacillales*
33	*Thiomonas* sp. enrichment culture clone 'DGGE gel band P1' (FJ628383)	81 %	*β-proteobacteria*
34	Uncultured bacterium clone F776O8Q01BGZHR (GU779167)	97 %	-
35	*Streptomyces* sp. (TB3481) (X76705)	80 %	*Actinobacteria*

-: unaffiliated group

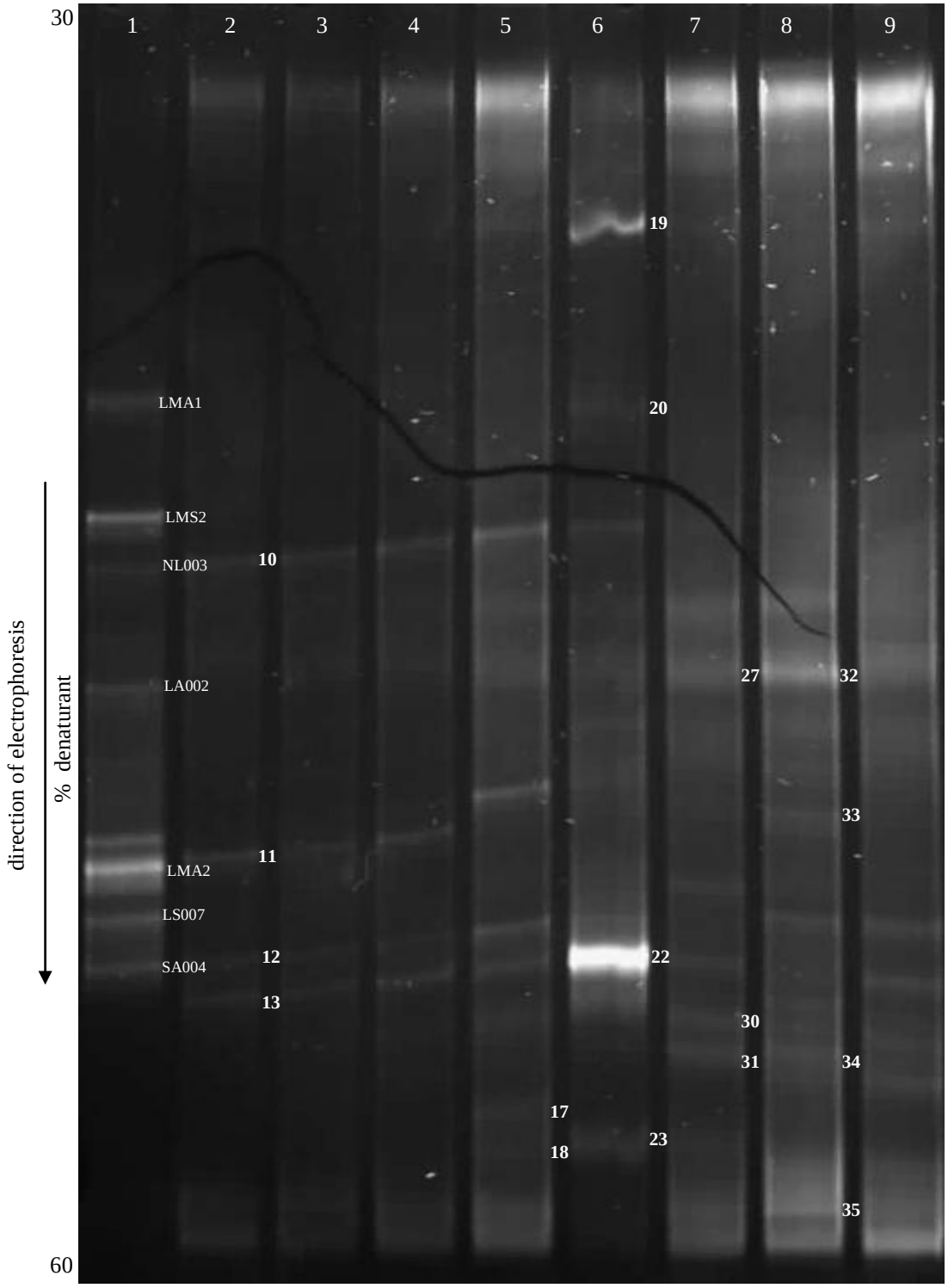

Figure 5.18: DGGE profile of biofilm samples after 20 weeks incubation in both the seawater/sediment system and loam soil (Lanes 1: DGGE marker; 2: autoclaved control in the seawater/sediment system; 3: non-autoclaved sample in the seawater/sediment system; 4: sodium nitrate in the seawater/sediment system; 5: ammonium nitrate in the seawater/sediment system; 6: autoclaved control in the loam soil; 7: non-autoclaved sample in the loam soil; 8: sodium nitrate in the loam soil and 9: ammonium nitrate in the loam soil). The numbers indicate gel portions that were excised for re-amplification and sequencing.

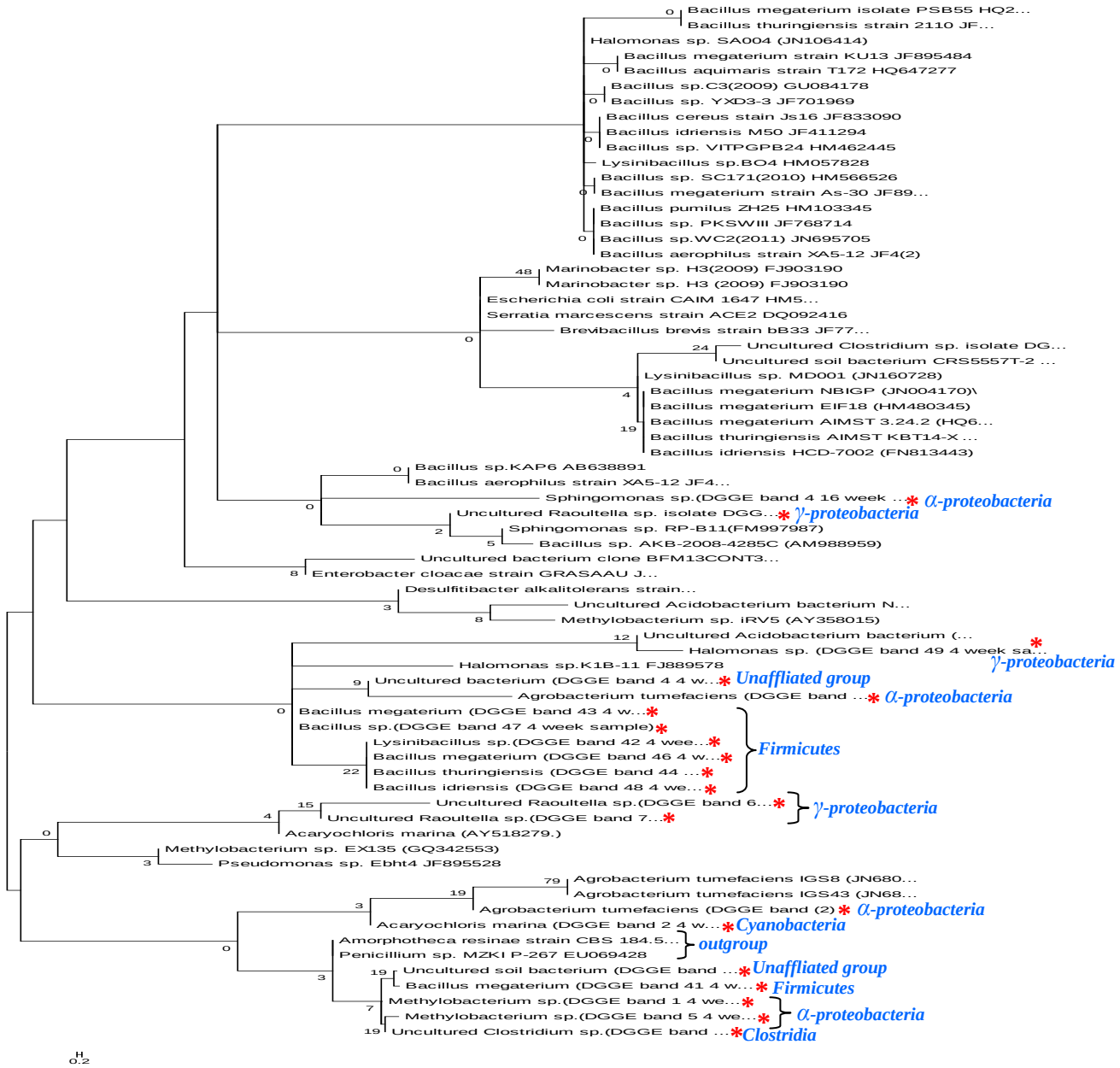

Figure 5.19: The evolutionary history was inferred using the Neighbor-Joining method for the non-autoclaved loam soil sample. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 110 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. (* indicates excised bands identified in this study)

Table 5.9 illustrates the bacterial species isolated from the extracted DGGE bands at the different sampling times. The bacterial isolates, *Acinetobacter calcoaceticus* and *Methylobacterium* sp. were detected in both the seawater/sediment and loam soil systems. *Bacillus* sp. was detected in both environmental systems throughout the study except at eight weeks of sampling. *Pseudomonas fluorescens* was present in both systems during the initial stages of the experiment however, this isolate was present only in the loam soil system after 20 weeks. *Mesorhizobium* sp. was present in both environmental systems throughout the study except that it was not detected at 12 weeks in the seawater/sediment system. In addition, *Lysinibacillus* sp. were present in both stimulated systems until 12 weeks of incubation.

Agrobacterium tumefaciens were present in both systems at eight and 20 weeks of incubation. *Rhodopseudomonas* sp. were present in both systems but were more prominent in the seawater/sediment system. The bacterial isolates, *Bradyrhizobium* sp. and *Streptomyces* sp. were detected in both environmental systems at the end of the study. During the initial stages of the experiment, *Clostridium thermopalmarium* was isolated from the non-autoclaved sample in the seawater/sediment system. *Marinobacter* sp. and *Sphingobacterium* sp. were detected in the autoclaved sample and the ammonium nitrate-treated seawater/sediment sample after 12 and 16 weeks of incubation respectively. At the end of the 20 week study period, *Micrococcus* sp. and *Thiomonas* sp. were only detected in the ammonium nitrate-treated seawater/sediment sample and the sodium nitrate-treated loam soil sample respectively.

Streptococcus thermophilus was detected in the loam soil sample supplemented with sodium nitrate after eight and 20 weeks of incubation. Although there were some common isolates over time in the systems, many dominant species were identified with most being isolated at the initial stages. *Terribacillus saccharophilus* and *Desulfohalobium retbaense* were isolated from the loam soil samples supplemented sodium nitrate at four weeks.

Table 5.9: Bacterial isolates extracted from DGGE gels during the study period in stimulated environmental systems

ISOLATES	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS
SEAWATER/SEDIMENT SYSTEM					
Acinetobacter calcoaceticus	X	X	X		X
Bacillus sp.	X		X	X	X
Pseudomonas fluorescens	X	X	X		
Mesorhizobium sp.	X	X		X	X
Lysinibacillus sp.	X	X	X		
Methylocystis minimus	X				
Chryseobacterium sp.	X	X			
Brevibacillus sp.	X		X		X
Roseobacter sp.	X				
Clostridium thermopalmarium	X				
Cohaesibacter sp.	X				
Nitratireductor kimnyeongensis		X			
Marinobacter sp.			X		
Burkholderia cepacia			X		
Sphingobacterium sp.				X	
Micrococcus sp.					X
Agrobacterium tumefaciens		X			X
Rhodopseudomonas sp.	X	X	X		
Methylobacterium sp.	X	X	X	X	X
Bradyrhizobium sp.					X
Streptomyces sp.					X
LOAM SOIL SYSTEM					
Acinetobacter calcoaceticus	X			X	X
Bacillus sp.	X		X	X	X
Pseudomonas fluorescens	X	X			X
Mesorhizobium sp.	X	X	X	X	X
Lysinibacillus sp.	X	X	X		
Agrobacterium tumefaciens		X			X
Streptococcus thermophilus		X			X
Rhodopseudomonas sp.		X			X
Sphingomonas sp.				X	X
Methylobacterium sp.	X	X	X	X	X
Acaryochloris marina	X				
Desulfohalobium retbaense	X				
Terribacillus saccharophilus	X				
Bacillus vireti	X				
Lactobacillus sakei		X			
Micromonospora sp.			X		
Bradyrhizobium sp.					X
Thiomonas sp.					X
Streptomyces sp.					X
Methylocystis minimum	X				
Chryseobacterium sp.		X			
Brevibacillus sp.	X		X		X

The phylotype profiles of the samples from both the seawater/sediment and loam soil systems were compared to their respective autoclaved controls by calculating the pairwise similarity coefficient,

Sorenson's Index (Table 5.10). There was a greater degree of similarity (*Cs* value of 0.8) between the samples and their autoclaved controls at week 4. At 16 weeks, a *Cs* value of 0 was observed for the loam soil samples, indicating that these samples were completely different from the autoclaved control. According to the *Cs* values in Table 5.10 it can be noted that the loam soil samples depict a lower degree of similarity to the autoclaved control than the samples from the stimulated seawater/sediment system.

Table 5.11 illustrates the similarity matrix between the seawater/sediment samples and the loam soil samples over time. A lower degree of similarity was observed in the autoclaved control between both samples at 16 and 20 weeks with a *Cs* value of 0.25 and 0.27 respectively. At 20 weeks, a *Cs* value of 0.33 and 0.46 was observed in the non-autoclaved sample and in the sample supplemented with sodium nitrate respectively, indicating a lower degree of similarity between the samples. In the sample with additional ammonium nitrate, a *Cs* value of 1 was observed at 16 weeks, indicating that the samples were identical between the loam soil and the seawater/sediment system.

Table 5.10: Similarity (C_s) matrix for the seawater/sediment system and loam soil experimental samples compared to the autoclaved control phylotype profiles

	SEAWATER/SEDIMENT SYSTEM			LOAM SOIL		
TIME (WEEKS)	NON-AUTOCLAVED	SODIUM NITRATE	AMMONIUM NITRATE	NON-AUTOCLAVED	SODIUM NITRATE	AMMONIUM NITRATE
4	0.8	0.87	0.8	0.8	0.82	0.88
8	0.9	0.9	0.9	0.5	0.4	0.18
12	0.75	0.75	0.57	0.57	0.57	0.25
16	0.22	0.66	0.66	0	0	0
20	0.9	0.9	0.53	0.375	0.24	0.14

Table 5.11: Similarity (C_s) matrix between the seawater/sediment system and loam soil phylotype profiles

TIME (WEEKS)	AUTOCLAVED CONTROL	NON-AUTOCLAVED	SODIUM NITRATE	AMMONIUM NITRATE
4	0.73	0.6	0.66	0.66
8	0.5	0.55	0.66	0.29
12	0.75	0.57	0.57	0.29
16	0.25	0.66	0.6	1
20	0.27	0.33	0.46	0.71

5.4 DISCUSSION

In this study FISH was carried out on biofilm scrapings with hybridized fluorescent oligonucleotide probes, EUB338, SRB385 and NON338 as a negative control. After 20 weeks of incubation, the total cell counts detected for SRB were lower in the samples supplemented with sodium nitrate in both the stimulated loam soil and the seawater/sediment systems. However, in the sample supplemented with ammonium nitrate, there was an increase in cell counts for SRB. The SRB385 probe is specific for sulphate reducers of the delta *Proteobacteria* and several Gram positive bacteria (e.g. *Clostridium* spp.) (Okabe *et al*., 1999). It is known that bacterial growth is stimulated by the addition of ammonium nitrate (Chu *et al*., 2005). Ammonium is incorporated into the cell material as organic nitrogen and is a nutritional supplement for many bacteria (Philips *et al*., 2002). The detection of the general bacterial population present in the biofilm samples showed an increase in cell counts in the samples with additional nitrates in the seawater/sediment system. The probe EUB338 revealed the presence of a higher abundance of more active bacteria, including SRB. However, it is noted that the specificity of the SRB385 probe for *in situ* hybridization is still problematic (Ito *et al*., 2002). It is understood that populations of viable but non-culturable cells accounts for much of the total bacterial population and activity *in situ* (Teske *et al*., 1996). The FISH counts were also associated with a relatively large standard deviation due to the heterogeneity of the biofilm. Low cell counts of SRB were observed using the probe SRB385. This is probably because of substrate diffusion limitation, presence of nitrate which created competition for organic matter with denitrifying bacteria, and oxygen inhibition (Okabe *et al*., 1999). It can also be speculated that the number of SRB cells were underestimated due to the presence of SRB species which cannot be detected by the probe SRB385.

There were similar counts of hybridized cells for both EUB and SRB probes in the non-autoclaved samples after 20 weeks of incubation in both the stimulated systems however, there was an increase in weight loss measured in this sample in the stimulated seawater/sediment system (7.09 mg/g) than that observed in the stimulated loam soil system (2.465 mg/g), as seen in Chapters 2 and 3 respectively. In the nitrate-treated systems, higher counts of positively hybridized cells were detected in the stimulated seawater/sediment system than the stimulated loam soil system. This is consistent with the weight loss measurement observed in the seawater/sediment system (22.49 mg/g and 20.97 mg/g for sodium nitrate and ammonium nitrate treated systems respectively) and in the loam soil system (26.735 mg/g and 4.36 mg/g for sodium nitrate and ammonium nitrate treated systems respectively).

In this study, DNA extracted from the biofilm developed on corroded mild steel coupons, was subjected to 16S rDNA PCR and subsequent DGGE analysis. The purpose of this technique is to determine the genetic diversity of natural microbial communities and identify dominant community members whose phylogenetic affiliations were subsequently determined by sequencing of DGGE fragments. The DGGE profiles showed multiple bands indicating the diversity of the bacterial population present in the biofilm sample. Increased number of DGGE bands was the result of the development of different micro-habitats within the biofilm. This could be a result of resistance to mass transfer and conversions, whereby microzonations develop within the biofilm, thus providing a broader range of niches for bacteria with different physiological characteristics (Santegoeds *et al*., 1998).

According to the study conducted by Murray *et al*. (1996), bands representing constituents amplified with high initial template DNA concentrations (50 ng) stained more intensely than constituents with 5 ng of template DNA. The authors suggested that while this pattern confers a relationship between band intensity and the relative abundance of DNA from that phylotype in the template mixture, variation in 16S rRNA gene copy numbers, PCR biases, or co-migration of bands from different phylotypes could also cause variation in band intensity.

The phylotype profiles of the samples obtained from the stimulated seawater/sediment and loam soil systems were compared by calculating the pairwise similarity coefficient, Sorenson's Index, *Cs*. The loam soil samples depicted a lower degree of similarity to its autoclaved control than that observed in the seawater/sediment samples. In the samples with additional ammonium nitrate in both environmental systems, a *Cs* value of 1 was obtained, indicating that there was identical bacterial isolates found in both the loam soil and the seawater/sediment system after 16 weeks of incubation. The additional ammonium nitrate stimulated the same bacterial population in both systems at that particular time. Many dominant bands were excised and sequenced. Sequencing techniques provided an analysis of the bacterial 16S rDNA gene composition present in the biofilms of mild steel samples. The microorganisms present in the biofilms belonged to seven different phylogenetic groups (Alpha-, Beta-, Gamma-, Delta-Proteobacteria, Actinobacteria, Lactobacillales and Firmicutes).

In this study it was observed that certain bacterial isolates such as *Bacillus* sp., *Acinetobacter calcoaceticus*, *Pseudomonas fluorescens*, *Lysinibacillus* sp. and *Mesorhizobium* sp. were common in the stimulated environmental systems over time. It is possible that these isolates were indigenous to the systems and therefore, better adapted to the environmental conditions however, some dominant bacterial species such as *Methylobacterium* sp., *Terribacillus saccharophilus*, *Chyrseobacterium* sp. and

Roseobacter sp. were also detected. In this case, the addition of nitrates may have stimulated the growth and metabolic activities of these microbial isolates. The dominant bacterial isolates detected after four weeks of the study were not detected at the end of the study. However, some isolates such as *Bradyrhizobium* sp., *Thiomonas* sp. and *Streptomyces* sp. were detected in the loam soil system at the end of the study. In addition, *Micrococcus* sp. was detected as a dominant bacterial isolate in the seawater/sediment system at the end of the study. These isolates were probably only detected at the end of the study because they required time for adaptation to the particular environments that they were exposed to.

Following sequencing, it could be noted that there was a presence of an anaerobic, Gram-negative, halophilic, SRB (*Desulfohalobium retbaense*) that was detected in the loam soil sample supplemented with sodium nitrate after four weeks of incubation. In addition, an aerobic, Gram-negative, nitrate-reducing marine bacterium (*Nitratireductor kimnyeongensis*) was detected in the ammonium nitrate treated seawater/sediment sample after eight weeks of incubation. The detection of these isolates could provide an informative indication that the additional nitrates did stimulate a population shift in the stimulated systems from SRB to NRB populations.

Although SRB were not isolated in this study by culturable techniques, low counts were detected by FISH using the SRB385 probe. It may be possible to predict their characteristics and metabolic capabilities based on comparison with phylogenetically related bacteria and then infer their possible participation in biofilm formation and biocorrosion processes. It is possible that SRB, while not necessarily abundant in the stimulated systems, are very aggressive and thus contribute to the corrosion process. Although only one isolate of SRB was detected from the DGGE analysis, it does correspond with other studies where DGGE has been shown only to detect PCR amplicons making up >1 % of the total pool of amplicons (i.e. community members making up >1 % of the total community) (Muyzer *et al*., 1993; Kjellerup *et al*., 2009). The short length of the amplicon obtained from primer pair 357F-GC and 518R, may limit the phylogenetic information contained in the sequenced bands. It has been shown previously however, that relatively short sequences, such as those obtained from DGGE analysis, are sufficient for an approximate phylogenetic identification (Kawai *et al*., 2002).

The presence of both aerobic and anaerobic organisms was detected in the biofilm samples following DGGE analysis. The genus *Sphingomonas* identified from the loam soil samples in this study have been previously identified in a wide variety of environments, including terrestrial and rhizosphere soil, marine, freshwaters and sediments. The locations have often been exposed to pollution and it has been shown that

Sphingomonas has the ability to degrade polycyclic aromatic hydrocarbons (PAHs), pesticides and nonylphenols (Kjellerup *et al*., 2005).

There were two phylotypes and isolates observed after four weeks of incubation that were affiliated with the anaerobic, Gram-positive class *Clostridia*, indicating the existence of reduced zones in the biofilm. The prevalence of this class might suggest that the ability to form spores is a selective advantage, for example, for survival in high temperature regions or during periods of low nutrient availability. Although low overall cell densities characterized the biofilms, they appear to constitute a heterogeneous environment allowing the establishment of metabolically and physiologically diverse bacterial communities (Kjeldsen *et al*., 2007).

Burkholderia isolated from the seawater/sediment system supplemented with sodium nitrate after 12 weeks of incubation, is considered to be a ubiquitous genus of soil bacterium related to PAH-degrading and herbicide-degrading processes. It has been classified as a microorganism potentially useful for bioremediation of several compounds and elements. Sequence analysis of the dominant bands derived from bacterial 16S rDNA gene amplicons were excised from the DGGE gels and indicated that the predominant bacteria found in the biofilms belonged to the *Bacillus* genera, suggesting that these may be the primary colonizers in biofilm formation. This genus has an impressive physiological diversity and its spores are highly resistant to unfavorable conditions. The *Bacillus* genus is resistant to gamma radiation, UV, H_2O_2, and desiccation. Some strains, and especially their spores, can bind irreversibly large amounts of metals and the presence of spores at advanced stages of colonization might account for the increasing radioactivity present in biofilm (Sarro *et al*., 2005). In addition to the primary colonizers, which may be the key players in the initial corrosion process, *Marinobacter* was also identified in the seawater/sediment sample supplemented with ammonium nitrate after 12 weeks of incubation. Members of this genus have been isolated from marine environments and are halophilic and nitrate reducers (Marques *et al*., 2012). This again indicates a population shift in the stimulated systems with the addition of nitrates.

Pseudomonads and *Acinetobacter* were also detected in the biofilms of corroded mild steel coupons. These bacteria have minimal nutritional requirements and are often present in aquatic environments that are rich in organic pollutants such as gasoline and solvents, etc. In addition, pseudomonads contribute to biofilm formation by producing exopolysaccharides and facilitating the attachment of other microorganisms and hence accelerate the corrosion process. Pseudomonads are also capable of both complete and incomplete denitrification (Zhu *et al*., 2003).

By using the molecular techniques described in this chapter, it is possible to follow community development and to detect the presence of SRB in complex biofilms. This would be difficult to achieve with conventional cultivation techniques such as that described in Chapter 4 however, the molecular techniques used in this study, were able to demonstrate the behaviour of a gradually changing, but complex microbial community. In this study it was clearly observed that the addition of nitrate did create a population shift from SRB to NRB population within the stimulated systems. It should be noted that more isolates of the respective populations could not be detected due to the concentration of nitrates as discussed in Chapters 2 and 3. Many of the microorganisms isolated in Chapter 4, especially those affiliated to the bacterial phyla Firmicutes and Gamma-Proteobacteria, were also detected using DGGE analysis. FISH allowed for an overall estimation of Eubacteria and SRB present in the biofilm on corroded mild steel coupons, indicating that the microbial population does play a role in the corrosion process. More specific techniques, perhaps involving the detection or expression of specific genes, are needed so that molecular techniques can be used as reliable diagnostic tools. In addition, further analysis of the biofilm samples and *in situ* hybridization with more specific probes might provide a more detailed picture of the abundance, distribution, and functioning of the SRB groups (Santegoeds *et al*., 1998).

CHAPTER SIX

DISCUSSION AND CONCLUDING REMARKS

MIC-based corrosion is extremely aggressive, leading to industrial system failures. This process is difficult to eliminate and may elevate into chronic maintenance and operational problems (Lane, 2005). Failure to adequately address the deteriorating infrastructure networks threatens our environment, public health and safety (Gomez-Alvarez *et al*., 2012). To understand biocorrosion, it is important to integrate chemical and physical phenomena with microbial physiology (Coester and Cloete, 2005). The presence of microorganisms on material surfaces can modify their chemistry and morphology, often promoting the establishment and/or maintenance of physico-chemical reactions not normally favored in the absence of the microbes and harmful to the performance and integrity of the material (Beech *et al*., 2005). In general, bacteria can oxidize a wide variety of chemicals and utilize it as a nutrient source. This in turn enhances the proliferation of bacteria. Bacteria with an aerobic metabolism, generally have a faster rate of development than anaerobic organisms, with a much higher metabolic activity. Consequently, these are organisms with greater potential in terms of the influence on the corrosion rate (Santana *et al*., 2012). The isolation and identification of microorganisms involved in the corrosion process is a necessary and mandatory step in order to design methods to prevent and control it (Correia *et al*., 2010). It is generally believed that the risk of corrosion may be induced with nitrate addition by microbial conversion of nitrate to nitrite (Halim *et al*., 2012).

In this study, similar weight loss measurements were observed on mild steel coupons in the nitrate-treated systems in both loam soil and the stimulated seawater/sediment systems. From the results obtained, it was observed that nitrate addition increased the weight loss measurements of both stainless and mild steel coupons over a 20 week period. This could be due to the stimulation of the metabolic activities of the corrosion-causing microorganisms by the additional nitrates, thus increasing the weight loss of the coupons. This observation can be supported by the SEM images depicting corrosion product build up, microbial adherence and colonization with biofilm formation.

EDX analyses of the mild steel coupons in both the stimulated loam soil and seawater/sediment systems revealed that the main components of the corrosion products were iron and oxygen as seen by the presence of the reddish-brown deposits observed in the stereo microscopic images. There were also trace quantities of calcium, silicon and manganese indicating the presence of iron-oxidizing bacteria that may significantly participate in corrosion deposit formation (Satheesh and Wesley, 2010). Samples of natural seawater, when stored, are known to change in corrosivity from the bulk seawater from which they were

taken. This is due in part to the fact that the minor constituents, including the living organisms and their dissolved organic nutrients, are in delicate balance in the natural environment. The aggressive corrosive characteristics have been attributed to the presence of other ions occurring in seawater, particularly Ca^{2+} and Mg^{2+}. The cathodic reduction of oxygen produces local alkaline surface conditions which precipitate $CaCO_3$ and $Mg(OH)_2$. These calcareous deposits are believed to promote a physical barrier against oxygen diffusion and thus decrease the corrosion rate. Cathodic protection of steel immersed in seawater is known to promote formation of these calcareous deposits (Moller *et al*., 2006).

It was also observed that the addition of nitrates reduced the weight loss measurements of mild steel coupons in conjunction with the bacteria isolated in this study. However, nitrate addition demonstrated differential effects on the weight loss measurements on the stainless steel coupons. It can be noted that certain consortia of microorganisms reduced the weight loss measurements of the mild steel coupons more so than that observed with the individual isolates. Isolation, identification and sequence analyses on the microorganisms revealed that the majority belonged to the *Bacillus* species affiliated with the bacterial phylum Firmicutes. In addition, *Psuedomonas* sp., *Marinobacter* sp., and *Halomonas* sp. were identified and were affiliated with the bacterial phylum Gamma-Proteobacteria. Phylogenetic analyses of the isolated organisms displayed the dominance of *Bacillus* sp. with the corrosion process of the metals. The bacteria, *Bacillus* is the predominant species in the corrosion processes and the development of this bacteria is enhanced in the presence of oxygen. However, when the oxygen tension on the metal surface decreases. the metabolism is now predominantly anaerobic and therefore, the combination with SRB has a synergic effect which favours corrosion through the alteration of the metal surfaces (Santana *et al*., 2012). The results show that the isolated aerobic microorganisms do play a role in the corrosion process in both stainless and mild steel coupons. However, it can be noted that certain isolated bacterial species have the potential of mitigating corrosion on the metal coupons.

It was observed in this study, that with increasing weight loss, there seems to be a decrease in the population growth of both the total heterotrophic and sulphate-reducing bacteria present on the mild steel surface in the stimulated seawater/sediment system using the MPN method. However, after 20 weeks of incubation in the stimulated loam soil system, an increase in sulphate-reducing and total heterotrophic bacterial populations occurred in the mild steel samples supplemented with nitrates. In principle, MPN counts give a more stringent quantitative picture of the distribution of SRB, since only viable and cultivable organisms are counted, no matter how small this population might be in comparison with other bacterial populations. The physiological selectivity of MPN counts however, is often regarded as an obstacle to the complete analysis of a microbial community. Therefore, it should be viewed as a specific

advantage and an important complement for rRNA-based molecular methods, which can, by their nature, give no direct information on the physiological capabilities of bacterial populations (Teske *et al*., 1996).

Two approaches, such as FISH and DGGE analysis, were used to investigate the population dynamics present within the biofilms on the surface of corroded mild steel coupons. FISH was carried out on biofilm scrapings off corroded mild steel coupons with hybridized fluorescent oligonucleotide probes, EUB338, SRB385 and NON338 as a negative control. In the nitrate-treated systems, higher counts of positively hybridized cells were detected in the stimulated seawater/sediment system compared to the loam soil system. After 20 weeks of incubation, the total cell counts detected for SRB were lower in the samples supplemented with sodium nitrate in both the stimulated seawater/sediment and loam soil systems. However, in the sample supplemented with ammonium nitrate, there was an increase in cell counts for SRB.

DGGE analsysis and subsequent sequencing of the excised bands, revealed that the dominant microorganisms present on the corroded mild steel coupons belonged to seven different phylogenetic groups (Alpha-, Beta-, Gamma-, and Delta-Proteobacteria; Actinobacterial; Lactobacillales and Firmicutes). The detection of a sulphate-reducing bacterium (*Desulfohalobium retbaense*) and nitrate-reducing bacteria (*Nitratireductor kimnyeongensis* and *Marinobacter* sp.) in the nitrate-treated systems revealed that the addition of nitrates stimulated a population shift from SRB to NRB populations. This is in accordance with the Biocompetitive Exclusion Strategy. These counts are corresponding with the MPN counts that show an increase in SRB population in the loam soil sample with additional ammonium nitrate. *In-situ* hybridizations depicted an increase in the general bacterial population in the seawater/sediment sample with additional nitrates. However, MPN analysis revealed a decline in total heterotrophic and SRB population in these samples.

The identification of SRB is not easy due to their diversity. They are adapted to a great variety of environments, but are mainly observed in seawater where they contribute to the mineralization of 50% of the organic matter (Rubio *et al*., 2005). However, unlike many obligate anaerobic bacteria, SRB are capable of limited oxygen metabolism (Barton and Tomei, 1995; Okabe *et al*., 1999). Other microbes besides SRB could possibly play significant roles in influencing corrosion. For instance, *Bacillus* sp. have been detected and isolated in water pipelines (Lopez *et al*., 2006), in galvanized steel (Bolton *et al*., 2010), in corrosive lesions at a Granite Mountain Record Vault (Kan *et al*., 2011), and in the current study. *Bacillus* species have been capable of ferrous/manganese oxidation (Rajasekar *et al*., 2007a), ferric reduction and acid production (Valencia-Cantero and Pena-Cabriales, 2003). As described in earlier

biocorrosion studies, the simultaneous presence of reduced sulphur compounds and oxygen can create a very corrosive environment because of the formation of elementary sulphur, polysulphides, and other compounds which can establish an autocatalytic corrosion process that can take place even in the absence of active SRB (Lee *et al*., 1993; Nielsen *et al*., 1993; Kjellerup *et al*., 2009). SRB arise from four different phylogenetic ancestries therefore several FISH probes are necessary to detect all SRB subgroups (de Jesus Santos *et al*., 2010), and this is lacking in this study.

From the results obtained in this study, it seems that the addition of nitrates did not significantly reduce the rates of corrosion of both the mild and stainless steels. However, it does seem that the environmental conditions posed as an important factor in the corrosion processes. This is seen in loam soil, where the rates of corrosion are reduced in stainless steel with the addition of nitrates. Further studies need to be conducted to analyse the environmental type and microbial composition as it is clear that the corrosion measurements may differ slightly in loam soil as compared to the seawater/sediment system. Thereafter, optimization of the concentration of nitrates needs to be conducted before implementation in the stimulated systems in order to test the effectiveness and possible potential for this mechanism to occur in the field. It is noteworthy to point out that the cultivable individual isolates, in conjunction with nitrates, seem to reduce the corrosion rates on mild steel. Biofilms growing on the surface of mild steel coupons were dominated by *Proteobacteria* with a putative aerobic metabolism, but also contained anaerobic organisms such as those affiliated to the Gram-positive class *Clostridia*. The current results indicate that the corrosion observed on the metal surface is dependent on size and abundance of aerobic and anaerobic microorganisms. These cultivable organisms, together with the non-cultivables such as those detected by molecular approaches, need to be tested further to analyse their potential in corrosion inhibition.

Mechanisms proposed as pertinent to biocorrosion reflect the range of physiological activities carried out by diverse types of microorganisms found within biofilms. These mechanisms vary with microbial species and the chemistry of the colonized metal surface (Beech and Sunner, 2004). An understanding, at the molecular level, of the interactions of microbial cells and their metabolic products, including EPS, with material surfaces is therefore of paramount importance in the prevention of biofouling and biocorrosion (Beech and Sunner, 2004; Beech *et al*., 2005). Many of the microorganisms and the general mechanism involved in MIC has been known for decades and recent studies using molecular approaches have more accurately described the microbial ecology of these engineered systems. However, a better understanding of the metabolic processes and functional capabilities is needed to develop new approaches to mitigate MIC and its associated effects (Gomez-Alvarez *et al*., 2012). Gaining deeper insight into the

fundamental mechanisms of biofilm-mediated deleterious interfacial processes will, undoubtedly, result in the development of practices that will aid in their control (Beech and Sunner, 2004; Beech *et al*., 2005).

The prevention and control of MIC may seem like a daunting task however, with knowledge of how and where MIC occurs, as well as the prevention and control methods that may be used, the majority of problems can be prevented. There is a need to implement a better means of destroying biofilms and also to develop cost-effective and environmentally friendly preventatives (Lane, 2005). It is possible that the use of corrosion inhibitors will have biocidal effects on bacteria however, bacteria are also well known for their ability to oxidize a wide variety of chemicals and use them as nutrients (i.e. sources of carbon and nitrogen) under a variety of conditions. Results from a recent study by Akpabio *et al*. (2011) concluded that pre-characterization of the MIC-microorganisms assists the engineer and scientist in the effective control of MIC by helping to select an inhibitor which will act as a biocide, not as a nutrient source.

To identify the biofilm bacteria involved in biocorrosion, a combination of *in situ* techniques such as FISH and microautoradiographic (MAR) might be used. According to the results obtained in this study, further analysis of the biofilm samples and *in situ* hybridization with more specific probes might provide a more detailed picture of the abundance, distribution and functioning of the SRB groups. Future method development of these techniques many allow a direct identification of the bacteria involved in corrosion processes as well as in reverse processes. Additional research could involve the application of the combined use of probes labeled with different fluorescent dyes to define spatial relationships among populations. Future studies may also focus on the importance of the correct choice of primer strategies, the requirement for screening and sequencing of non-target organisms and the requirement for multiple approaches, particularly during investigations of previously unstudied environments. For characterization of environmental samples, another useful approach other than FISH and DGGE which were conducted during this study, is the use of microarrays to detect 16S rRNA or its genes. Currently, microarrays are being used to characterize microbial populations and to detect microorganisms in complex environments.

Another promising approach in corrosion studies is metagenomic analyses of microbial communities in biofilms on corroded metals (Gomez-Alvarez *et al*., 2012). There are ongoing studies that aim for the isolation and characterization of anaerobes which were partially initiated by metagenomic studies and considers their important role in the biocorrosion processes (Correia *et al*., 2010). Metagenomic studies enables detection, identification and reconstruction of metabolic pathways involved in MIC. The information generated from the sequencing libraries will allow understanding of the genetic network and

microbial members involved in the biofilms formed on corroded metals. The information is also relevant to track microbial populations associated with biofilms and to evaluate molecular assays used to detect key functional genes. However, a relatively low number of annotated genes is common in metagenomic studies and is primarily due to the relatively small and biased diversity of genomes sequenced, novel genes yet to be placed in functional groups, and sequencing and processing errors. For diverse and not well understood systems, annotation of gene functions can also be limited by the extent of the database of previously sequenced and characterized genes (Gomez-Alvarez *et al*., 2012).

MIC is a matter of great concern all over the world. The costs associated with prevention of the corrosion process and replacement of deteriorated metal structures are economically stifling. Researchers and engineers are continuously in search of cost-effective means to prevent the corrosion of steel for the duration of a structure's design life (Abdulrahman *et al*., 2011). To optimize MIC prevention and control in national and international industries, collaboration between engineers and microbiologists will be advantageous to develop a more environmentally sound and potentially cost-effective approach based on nutrient addition to control corrosion of metals.

REFERENCES

Abiola, O.K., and N.C. Oforka. 2004. Studies on the inhibition of mild steel corrosion by 1- Phenyl-3-methylpyrazol-5-one in hydrochloric acid (HCl) solution. *The Journal of Corrosion Science and Engineering*. **7** (2): 1 – 15.

Abdulrahman, A.S., M. Ismail and M.S. Hussain. 2011. Corrosion inhibitors for steel reinforcement in concrete: A review. *Scientific Research and Essays*. **6** (20): 4152-4162.

Adeosun, S.O., E.I. Akpan and S.A. Balogun. 2012. Wrought aluminium alloy corrosion propensity in domestic food cooking environment. *International Scholarly Research Network*. doi:10.5402/2012/432342.

Akpabio, E.J., E.J. Akott and M.E. Akpan. 2011. Inhibition and control of Microbiologically Influenced Corrosion in oilfield materials. *Environmental Research Journals*. **5** (2): 59 - 65.

Al-Faiz, M.Z. and L.S. Mezher. 2012. Cathodic protection remote monitoring based on wireless sensor network. *Wireless Sensor Network*. **4**: 226-233.

AlHazzaa, M.I. 2007. A comparative study of soil corrosivity of the university compass. King Saud University, College of Engineering, Research Center. Final Research Report No. 45/426.

Allam, L.M., J.S. Arlow and H. Saricimen. 1991. Initial stages of atmospheric corrosion of steel in the Arabian Gulf. *Corrosion Science*. **32**: 417 – 432.

Al-Sultani, K.F. and J.N. Nabat. 2012. Protect of underground oil pipelines by using (Al-Sn-Zn) as sacrificial anode in Al-Qasim region. *Journal of American Science*. **8** (6): 158 – 165.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *Molecular Biology*. **215**: 403-410.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new approach of protein database search programs. *Nucleic Acids Research*. **25**: 3389 - 3402.

Amann, R.I., J. Stromley, R. Devereux, R. Key and D.A. Stahl. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Applied and Environmental Microbiology*. **58** (2): 614-623.

Anandkumar, B., R.P. George, S. Tamilvani, N. Padhy and U.K. Mudali. 2011. Studies on microbiologically influenced corrosion of SS304 by a novel manganese oxidiser, *Bacillus flexus*. *Biofouling*. **27** (6): 675 - 683.

Angell, P. 1999. Understanding microbially influenced corrosion as biofilm-mediated changes in surface chemistry. *Current Opinion in Biotechnology*. **10**: 269 – 272.

Angell, P. and D.C. White. 1995. Is metabolic activity by biofilms with sulfate-reducing bacterial consortia essential for long-term propagation of pitting corrosion of stainless steel? *Journal of Industrial Microbiology*. **15**: 329 – 332.

Angell, P. and K. Urbanic. 2000. Sulphate-reducing bacterial activity as a parameter to predict localized corrosion of stainless alloys. *Corrosion Science*. **42**: 897 – 912.

Aramide, F.O. 2009. Corrosion inhibition of AISI/SAE steel in a marine environment. *Leonardo Journal of Sciences*. **15**: 47 - 52.

Atta, N.F., A.M. Fekry and H.M. Hassaneen. 2011. Corrosion inhibition, hydrogen evolution and antibacterial properties of new synthesized organic inhibitors on 316L stainless steel alloy in acid medium. *International Journal of Hydrogen Energy*, doi:10.1016/j.ijhydene.2011.02.134.

Augustin, M. and T. Ali-Vehmas. 2004. Assessment of enzymatic cleaning agents and disinfectants against bacterial biofilms. *Journal of Pharmacy and Pharmaceutical Sciences*. **7** (1): 55 – 64.

Aung, N.N., and Y.-J. Tan. 2004. A new method of studying buried steel corrosion and its inhibition using the wire beam electrode. *Corrosion Science*. **46**: 3057-3067.

Azumi, K., N. Yasui and M. Seo. 2000. Changes in the properties of anodic oxide films formed on titanium during long-term immersion in deaerated neutral solutions. *Corrosion Science*. **42**: 885 – 896.

Bano, A.S. and J.I. Qazi. 2011. Soil buried mild steel corrosion by *Bacillus cereus*-SNB4 and its inhibition by *Bacillus thuringiensis*-SN8. *Pakistan Journal of Zoology*. **43** (3): 555 – 562.

Bano, A.S. and J.I. Qazi. 2012. Corrosion and its control of soil buried mild steel coupons by *Paenibacillus dendritiformis*-MB14 and its antagonistic Bacillus licheniformis-MB6. *New Horizion in Science and Technology*. **1** (4): 100-105.

Barnes, L.M., M.F. Lo, M.R. Adams and A.H.L. Chamberlain. 1999. Effect of milk proteins on adhesion of bacteria to stainless steel surfaces. *Applied and Environmental Microbiology*. **65** (10): 4543 – 4548.

Barton, L.L., and F.A. Tomei. 1995. Characteristics and activities of sulfate-reducing bacteria. Biotechnology handbooks. Vol. 8. Series editors: T. Atkinson and R.F. Sherwood. Sulfate-Reducing Bacteria. L.L. Barton. Plenum Press. New York. 1-32.

Barton, L.L. and G.D. Fauque. 2009. Chapter 2 Biochemistry, Physiology and Biotechnology of Sulfate-Reducing Bacteria. *Advances in Applied Microbiology*. **68**: 41 – 98.

Beech, I.B. and C.W.S. Cheung. 1995. Interactions of exopolymers produced by sulphate-reducing bacteria with metal ions. *International Biodeterioration and Biodegradation*. **35**: 59 – 72.

Beech, I.B. 1996. The potential use of atomic force microscopy for studying corrosion of metals in the presence of bacterial biofilms – an overview. *International Biodeterioration and Biodegradation*. 141 – 149.

Beech, I.B. and C.C. Gaylarde. 1999. Recent advances in the study of biocorrosion – an overview. *Revista de Microbiologia*. **30**: 177-190.

Beech, I., A. Bergel, A. Mollica, Hans-Curt Flemming, V. Scotto and W. Sand. 2000. Simple methods for the investigation of the role of biofilms in corrosion. *Brite Euram Thematic network on MIC of industrial materials*. 1 - 27.

Beech, I.B. 2003. Sulfate-reducing bacteria in biofilms on metallic materials and corrosion. *Microbiology Today*. **30**: 115 – 117.

Beech, I.B. 2004. Corrosion of technical materials in the presence of biofilms – current understanding and state-of-the-art methods of study. *International Biodeterioration and Biodegradation*. **53**: 177 – 183.

Beech, I.B. and J. Sunner. 2004. Biocorrosion: towards understanding interactions between biofilms and metals. *Current Opinion in Biotechnology*. **15**: 181 – 186.

Beech, I.B., J.A. Sunner and K. Hiraoka. 2005. Microbe-surface interactions in biofouling and biocorrosion processes. *International Microbiology*. **8**: 157 – 168.

Bermont-Bouis, D., M. Janvier, P.A.D. Grimont, I. Dupont and T. Vallaeys. 2007. Both sulfate-reducing bacteria and Enterobacteriaceae take part in marine biocorrosion of carbon steel. *Journal of Applied Microbiology*. **102**: 161 - 168.

Blustein, G., J. Rodriguez, R. Romanogli, and C.F. Zinola. 2005. Inhibition of steel corrosion by calcium benzoate adsorption in nitrate solutions. *Corrosion Science*. **47**: 369 – 383.

Bodtker, G., T. Thorstenson, B. –L.P. Lillebo, B.E. Thobjornsen, R.H. Ulvoen, E. Sunde and T. Torsvik. 2008. The effect of long-term nitrate treatment on SRB activity, corrosion rate and bacterial community composition in offshore H_2O injection systems. *Journal of Industrial Microbiology and Biotechnology*. **35** (12): 1625 – 1636.

Bogan, B.W., B.M. Lamb and J.J. Kilbane 11. 2004. Development of an environmentally benign microbial inhibitor to control internal pipeline corrosion. *Gas Technology Institute*. 1 – 23.

Bolton, N., M. Critchley, R. Fabien, N. Cromar and H. Fallowfield. 2010. Microbially influenced corrosion of galvanized steel pipes in aerobic water systems. *Journal of Applied Microbiology*. **109**: 239 - 247.

Bonin, P.C., V.D. Michotey, A. Mouzdahir and J.F. Rontani. 2002. Anaerobic biodegradation of squalene: using DGGE to monitor the isolation of denitrifying bacteria taken form enrichment cultures. *FEMS Microbiology and Ecology*. **42**: 37 - 49.

Borenstein, S.W. 1994. *Microbiologically Influenced Corrosion Handbook*, Industrial Press Inc., New York.

Boyd, W.K. and F.W. Fink. 1978. Corrosion of metals in marine environments. Metals and Ceramics Information Center, Columbus. 1 – 62.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248 – 254.

Bragg, T. 2008. Corrosion of helical anchors in soil. *Pacific Housing Systems Inc.*

Brock, T.D. 1987. The study of microorganisms *in situ*: progress and problems. *A Symposium of the Society for General Microbiology*. **41**: 1 – 17.

Burstein, G.T. 2004. Electrochemistry of pit formation and growth. *Proceedings of Electrochemistry Society*. **19**: 1 – 12.

Castaneda, H., and X.D. Benetton. 2008. SRB-biofilm influence in active corrosion sites formed at steel-electrolyte interface when exposed to artificial seawater conditions. *Corrosion Science*. **50**: 1169 – 1183.

Castano, J.G., C.A. Botero, A.H. Restrepo, E.A. Agudelo, E. Correa and F. Echeverria. 2010. Atmospheric corrosion of carbon steel in Colombia. *Corrosion Science*. **52**: 216 – 213.

Chamritski, I.G., G.R. Burns, B.J. Webster and N.J. Laycock. 2004. Effect of iron-oxidizing bacteria on pitting of stainless steel. *Corrosion*. **60** (7): 658 – 669.

Chandy, J.P., and M.L. Angles. 2001. Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Research*. **35** (11): 2677 - 2683.

Characklis, W.G. and P.A. Wilderer. 1989. Structure and function of biofilms. John Wiley and Sons, Chichester. *Life Sciences Research Reports 46*. 1-17.

Chaves, I.A., and R.E. Melchers. 2011. Pitting corrosion in pipeline steel weld zones. *Corrosion Science*, doi:10.1016/j.corsci.2011.08.005

Chen, Y.C., C.M. Lee, S.K. Yen and S.D. Chyou. 2007. The effect of denitrifying Fe-oxidizing bacteria TPH-7 on corrosion inhibition of sodium molybdate. *Corrosion Science*. **49**: 3917-3925.

Chongdar, S., G. Gunasekaran and P. Kumar. 2005. Corrosion inhibition of mild steel by aerobic biofilm. *Electrochimica Acta*. **50**: 4655-4665.

Chu, C., C. Lu and C. Lee. 2005. Effects of inorganic nutrients on the regrowth of heterotrophic bacteria in drinking water distribution systems. *Journal of Environmental Management*. **74**: 255 – 263.

Clarridge, J.E.III. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Reviews.* **17** (4): 840 – 862.

Cloete, T.E., L. Jacobs and V.S. Brozel. 1998. The chemical control of biofouling in industrial water systems. *Biodegradation*. **9**: 23 - 37.

Coetser, S.E. and T.E. Cloete. 2005. Biofouling and biocorrosion in industrial water systems. *Critical reviews in Microbiology*. **31** (4): 213-232.

Colavita, M. 2000. Occurrence of corrosion in Airframes. *Paper presented at the RTO AVT lecture series on "Aging Aircraft Fleets: Structural and other subsystem aspects*. 6-1 – 6-10.

Compere, C., M.N. Bellon-Fontaine, P. Bertrand, D. Costa and P. Marcus. 2001. Kinetics of conditioning layer formation on stainless steel immersed in seawater. *Biofouling.* **17**: 129 - 145.

Compton, K.G. 1971. Seawater Tests. Handbook on Corrosion testing and evaluation. W.H. Ailor, Ed., John Wiley and Sons, New York. 507 – 514.

Correia, A.F., J.F.O. Segovia, R.M. Bezerra, M.C.A. Goncalves, S.S. Ornelas, D. Silveira, J.C.T. Carvalho, S.P.S.S. Diniz and L.I.B. Kanzaki. 2010. Aerobic and facultative microorganisms isolated from corroded metallic structures in a hydroelectric power unit in the Amazon Region of Brazil. *Air, Soil and Water Research*. **3**: 113 – 121.

Costerton, J.W., Z. Lewandowski, D.E. Coldwell, D.R. Korber and H.M. Lappin-Scott. 1995. Microbial biofilms. *Annual Review of Microbiology*. **49**: 711 – 745.

Creepi, B.J. 2001. The evolution of social behavior in microorganisms. *TRENDS in Ecology and Evolution*. **16** (4): 178 – 183.

Cristobal, A.B., M.A. Arenas, A. Conde and J. de Damborenea. 2006. Corrosion of stainless steels covered by exopolymers. *Electrochimica Acta*. **52**: 546-551.

Cunat, P.J. 2001. Corrosion resistance of stainless steels in soils and in concrete. Paper presented at the Plenary days of the Committee on the study of pipe corrosion and protection. 1 – 12.

Davey, M.E. and G.A. O'toole. 2000. Microbial biofilms: from Ecology to Molecular Genetics. *Microbiology and Molecular Biology Reviews*. **64** (4): 847 – 867.

Davis, J.R. 1987. Corrosion in specific industries and environments. Metals Handbook: Corrosion. 9th Edition, Vol. 13. ASM International, Metals Park. 893 – 926.

Dayal, H.M., B.K. Gupta, K.C. Tewari, K. Mehta, Chandrashekhar and S.K. Gupta. 1988. Underground corrosion by microorganisms Part 1: Analytical studies of some Indian soils. *Defense Science Journal*. **38** (2): 209-216.

de Carvalho, C.C.C.R. 2007. Biofilms: Recent developments on an old battle. *Recent patents on biotechnology*. **1**: 49-57.

de Jesus Santos, A.F., L.L.F Batista, J.B.T. Lima, R.C. de Castro Almeida, M.R. de Abreu Roque, E.R. de Souza and P.F. de Almeida. 2010. Evaluation of the Fluorescence *In Situ* Hybridization Technique for the detection of Eubacteria and Sulfate-Reducing Bacteria from samples of water in oil fields. *Chemical Engineering Transactions*. **20**: 139 – 144.

Dexter, S.J. and C. Culberson. 1980. Global variability of natural seawater. *Materials Performance*. **19** (9): 16.

Dhanasekaran, D., N. Thajuddin, M. Rashmi, T.L. Deepika and M. Gunasekaran. 2009. Screening of biofouling activity in marine bacterial isolate from ship hull. *International Journal of Environmental Science and Technology*. **6**(2): 197 – 202.

Dickinson, W.H., F. Caccavo Jr, B. Olesen and Z. Lewandowski. 1997. Ennoblement of stainless steel by the manganese-depositing bacterium *Leptothrix discophora*. *Applied and Environmental Microbiology*. **63** (7): 2502-2506.

Dinh, H.T., J. Kuever, M. Mussmann, A.W. Hassel, M. Stratmann and F. Widdel. 2004. Iron corrosion by novel anaerobic microorganisms. *Nature*. **427**(6977): 829 – 832.

Drysdale, G.D., H.C. Kasan and F. Bux. 1999. Denitrification by heterotrophic bacteria during activated sludge treatment. *Water SA*. **25**: 357 – 362.

D'Souza, F. and N.B. Bhosle. 2003. Analysis of microfouling products formed on metallic surfaces exposed in a marine environment. *Biofouling*. **19**: 95 - 107.

D'Souza, F., A. Garg and N.B. Bhosle. 2005. Seasonal variation in the chemical composition and carbohydrate signature compounds of biofilm. *Aquatic Microbial Ecology*. **41**: 199 - 207.

Dubiel, M., C.H. Hsu, C.C. Chien, F. Mansfeld and D.K. Newman. 2002. Microbial iron respiration can protect steel from corrosion. *Applied and Environmental Microbiology*. **68** (3): 1440-1445.

Dubey, R.S. and S.N. Upadhyay. 2001. Microbial corrosion monitoring by an amperometric microbial biosensor developed using whole cell of *Pseudomonas* sp. *Biosensors and Bioelectronics*. **16**: 995-1000.

Dunne, W.M. Jr. 2002. Bacterial Adhesion: Seen any good biofilms lately? *Clinical Microbiology Reviews*. **15** (2): 155 – 166.

Dunsmore, B., J. Youldon, D.R. Thrasher and I. Vance. 2006. Effects of nitrate treatment on a mixed species, oil field microbial biofilm. *Journal of Industrial Microbiology and Biotechnology*. **33**: 454 - 462.

Eckford, R.E. and P.M. Fedorak. 2002. Planktonic nitrate-reducing bacteria and sulfate-reducing bacteria in some Western Canadian oil field waters. *Journal of Industrial Microbiology and Biotechnology*. **29** (2): 83 – 92.

Edyvean, R.G.J. and H.A. Videla. 1991. Biological Corrosion. *Interdisciplinary Science Reviews*. **16**: 267 – 282.

Ekine, A.S. and G.O. Emujakporue. 2012. Investigation of corrosion of buried oil pipeline by the electrical geophysical methods. *Environmental Research Journal*. **6** (1): 19 – 21.

El-Abed, S., S.K. Ibnsouda, H. Latrache and F. Hamadi. 2012. Scanning Electron Microscopy (SEM) and Environmental SEM: Suitable tools for study of adhesion stage and biofilm formation, Scanning Electron Microscopy, Dr Viacheslav Kazmiruk (Ed.), ISBN:978-953-51-0092-8, In Tech.

El-Meligi, A.A. 2010. Corrosion Preventive Strategies as a crucial need for decreasing environmental pollution and saving economics. *Recent Patents on Corrosion Science*. **2**: 22 – 33.

El-Shamy, A.M, T.Y. Soror, H.A. El-Dahan, E.A. Ghazy and A.F. Eweas. 2009. Microbial corrosion inhibition of mild steel in salty water environment. *Materials Chemistry and Physics*. **114**: 156 – 159.

Else, T.A., C.R. Pantle and P.S. Amy. 2003. Boundaries for biofilm formation: humidity and temperature. *Applied and Environmental Microbiology*. **69** (8): 5006 – 5010.

Enning, D., H. Venzlaff, J. Garrelfs, H.T. Dinh, V. Meyer, K. Mayrhofer, A.W. Hassel, M. Stratmann and F. Widdel. 2012. Marine sulfate-reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. *Environmental Microbiology*. **14** (7): 1772 – 1787.

Fadare, D.A, T.G. Fadara and O.Y. Akanbi. 2011. Effect of heat treatment on mechanical properties and microstructure of NST37-2 steel. *Journal of Minerals and Materials Characterization and Engineering*. **10** (3): 299 – 308.

Faimali, M., F. Garaventa, A. Terlizzi, M. Chiantore, R. Cattaneo-Vietti. 2004. The interplay of substrate nature and biofilm formation in regulating *Balanus amphirite* Darwin, 1854 larval settlement. *Journal of Experimental Marine Biology and Ecology*. **306**: 37 - 50.

Fang, H.H.P., Li-Chong Xu and Kwong-Yu Chan. 2002. Effects of toxic metals and chemicals on biofilm and biocorrosion. *Water Research*. **36**: 4709 – 4716.

Farro, N.W., L. Veleva and P. Aguilar. 2009. Copper Marine Corrosion: I. Corrosion rates in atmospheric and seawater environment of Peruvian Port. *The Open Corrosion Journal*. **2**: 130-138.

Feio, M.J., I.B. Beech, M. Carepo, J.M. Lopes, C.W.S. Cheung, R. Franco, J. Guezennec, J.R. Smith, J.I. Mitchell, J.J.G. Moura and A.R. Lino. 1998. Isolation and characterization of a novel sulphate-reducing bacterium of the *Desulfovibrio* genus. *Anaerobe*. **4**: 117 – 130.

Feio, M. J., V. Rainha, M. A. Reis, A. R. Lino and I. T. E. Fonseca. 2000. The influence of *Desulfovibrio desulfricans* 14 ATCC 27774 on the corrosion of mild steel. *Materials and Corrosion.* **51**: 69 1-697.

Flemming, H.-C, and H. Ridgway. 2008. Biofilm control: Conventional and Alternative Approaches. doi: 10.1007/7142_2008_20: 103 - 117.

Fonseca, Ines T.E., M. Jose Feio, Ana R. Lino and Valter L. Rainha. 1997. Biocorrosion of mild steel by SRB: Electrochemical studies. *Journal of Brazilian Chem. Soc.* **8** (2): 131- 135.

Fonseca, Ines T.E., M. Jose Feio, Ana R. Lino and Valter L. Rainha. 1998. The influence of the media on the corrosion of mild steel by Desulfovibrio desulfuricans bacteria: an electrochemical study. *Electrochimica Acta.* **43** (1-2): 213- 222.

Fontana, M.G. and N.D. Greene. 1967. Corrosion Engineering. McGraw-Hill, New York, New York.

Francisco, J.A. Nascimento, J. Naslund and R. Elmgren. 2012. Meiofauna enhances organic matter mineralization in soft sediment ecosystems. *Limnology and Oceanography*. **57** (1): 338 – 346.

Franklin, M.J., D.C. White and H.S. Isaacs. 1991. *Corrosion Science*. **32** (9): 945 - 952.

Freschi, C.R., L.F. de Oliveira e Silva Carvalho and C.J.B. de Oliveira. 2005. Comparison of DNA-extraction methods and selective enrichment broths on the detection of *Salmonella typhimurium* in swine feces by Polymerase Chain Reaction (PCR). *Brazilian Journal of Microbiology*. **36**: 363 - 367.

Garces, P., P.Saura, E.Zornoza and C.Andrade. 2011. Influence of pH on the nitrite corrosion inhibition of reinforcing steel in simulating concrete pore solution. *Corrosion Science*, doi: 10.1016/j.corsci.2011.08.002

Garcia, F., and A.L.R. Lopez. 2012. Corrosion inhibition in copper by isolated baccteria. *Anti-Corrosion Methods and Materials*. **59** (1): 10 - 17.

Gardner, L.R. and P.S. Stewart. 2002. Action of glutaraldehyde and nitrite against sulfate-reducing bacterial biofilms. *Journal of Industrial Microbiology and Biotechnology*. **29**: 354 – 360.

Geesey, G.G., R.J. Gillis, R. Avci, D. Daly, M. Hamilton, P. Shope and G. Harkin. 1996. The influence of surface features on bacterial colonization and subsequent substratum chemical changes of 316L stainless steel. *Corrosion Science*. **38** (1): 73 – 95.

Geesey, G. and M. Phinney. 1997. Center for Biofilm Engineering. Montana State University. (http://www.biofilm.montana.edu)

Gevertz, D., A. J. Telang, G. Voordouw, and G. E. Jenneman. 2000. Isolation and characterization of strains CVO and FWKO B, two novel nitrate-reducing, sulfide-oxidizing bacteria isolated from oil field brine. *Applied and Environmental Microbiology*. **66** (6): 2491–2501.

Gibson, G.R. 1990. Physiology and ecology of the sulphate-reducing bacteria. *Journal of Applied Bacteriology*. **69**: 769 – 797.

Gittel, A., K.B. Sorenson, T.L. Skovhus, K. Ingvorsen and A. Schramm. 2009. Prokaryotic community structure and sulfate reducer activity in water from high-temperature oil reservoirs with and without nitrate treatment. *Applied and Environmental Microbiology*. **75** (22): 7086 - 7096.

Gomez-Alvarez, V., R.P. Revetta and J.W.S. Domingo. 2012. Metagenome analyses of corroded concrete wastewater pipe biofilms reveal a complex microbial system. *BMC Microbiology*. **12**: 122-136.

Gonzalez-Rodriguez, C.A., F.J. Rodriguez-Gomez and J. Genesca-Llongueras. 2008. The influence of *Desulfovibrio vulgaris* on the efficiency of imidazoline as a corrosion inhibitor on low-carbon in seawater. *Electrochemical Acta*. **54**: 86 – 90.

Graves, J.W. and E.H. Sullivan. 1996. Internal corrosion in gas gathering system and transmission lines. *Materials Protection*. **5**: 33 – 37.

Greene, E.A., V. Brunelle, G.E. Jenneman and G. Voordouw. 2006. Synergistic inhibition of microbial sulfide production by combinations of the metabolic inhibitor nitrite and biocides. *Applied and Environmental Microbiology*. **72** (12): 7897 – 7901.

Griffin, R.B. 1987. Marine Atmosphere. Metals Handbook: Corrosion. Metals Handbook: Corrosion. 9th Edition, Vol. 73. ASM International, Materials Park, OH. 902 – 906.

Grigoryan, A.A., S.L. Cornish, B. Buziak, S. Lin, A. Cavallaro, J.J. Arensdorf and G. Voordouw. 2008. Competitive oxidation of volatile fatty acids by sulfate- and nitrate-reducing bacteria from an Oil field in Argentina. *Applied and Environmental Microbiology*. **74** (14): 4324-4335.

Gu, J-Dong. 2003. Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. *International Biodeterioration and Biodegradation*. **52**: 69 – 91.

Guiamet, P.S., S.G. Gomez de Saravia and H.A. Videla. 1999. An innovative method for preventing biocorrosion through microbial adhesion inhibition. *International Biodeterioration and Biodegradation*. **43**: 31 – 35.

Guiamet, P.S. and S.G. Gomez de Saravia. 2005. Laboratory studies of biocorrosion control using traditional and environmentally friendly biocides: An overview. *Latin American Applied Research*. **35**: 295 – 300.

Gurappa, I. 2002. Characterization of different materials for corrosion resistance under simulated body fluid conditions. *Materials Characterization*. **49**: 73 – 79.

Gounot, A. M. 1994. Microbial oxidation and reduction of manganese: consequences in groundwater and applications. *FEMS Microbiology Reviews*. **14**: 339-350.

Halim, A., E. Watkin and R. Gubner. 2012. Short term corrosion monitoring of carbon steel by biocompetitive exclusion of thermophilic sulphate-reducing bacteria and nitrate reducing bacteria. *Electrochimica Acta*. **77**: 348 – 362.

Han, W., G. Yu, Z. Wang and J. Wang. 2007. Characterisation of initial atmospheric corrosion carbon steels by field exposure and laboratory simulation. *Corrosion Science*. **49**: 2920 – 2935.

Haouri, O., M.-L. Fardeau, L. Casalot, J.-L. Tholozan, M. Hamdi and B. Ollivier. 2006. Isolation of sulfate-reducing bacteria from Tunisian marine sediments and description of *Desulfovibrio bizertensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. **56**: 2909 – 2913.

Harmsen, H.J.M., D. Prieur and C. Jeanthon. 1997. Group-Specific 16S rRNA-targeted oligonucleotide probes to identify thermophilic bacteria in marine hydrothermal vents. *Applied and Environmental Microbiology*. **63** (10): 4061 - 4068.

Harris, J.O. 1960. Soil microorganisms in relation to cathodically protected pipe. *Corrosion*. **16**: 441 – 448.

He, Q., K.H. Huang, Z. He, E.J. Alm, M.W. Fields, T.C. Hazen, A.P. Arkin, J.D. Wall and J. Zhou. 2006. Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough, inferred from global transcriptional analysis. *Applied and Environmental Microbiology*. **72** (6): 4370 – 4381.

Hernandez-Gayoso, M.J., G.Z. Olivares, N.R. Ordaz, C.J. Ramirez, R.G. Esquivel and A.P. Viveros. 2004. Microbial consortium influence upon steel corrosion rate, using polarization resistance and electrochemical noise techniques. *Electrochimica Acta*. **49**: 4295 – 4301.

Hesham, A.E.-L., and S.A. Alamri. 2012. Application of fluorescence *in situ* hybridization (FISH) to the analysis of sulfate reducing bacterial community in an oily bench scale reactor. *African Journal of Biotechnology*. **11** (44): 10221 - 10226.

Hitzman, D.O. and D.M.Dennis. 1997. Sulfide removal and prevention in gas wells. In *Proceedings Volume*. SPE Prod Oper Symp (Oklahoma City, OK, 3/9-3/11), 433-438.

Hitzman, D.O. and D.M. Dennis. 1998. Sulfide removal and prevention in gas wells. SPE paper 50980.

Hubert, C., M. Nemati, G. Jenneman and G. Voordouw. 2003. Containment of biogenic sulfide production in continuous up-flow packed-bed bioreactors with nitrate and nitrite. *Biotechnology Progress*. **19**: 338 – 345.

Hubert, C., N. Mehdi, G. Jenneman, G. Voordouw. 2005. Corrosion risk associated with microbial souring control using nitrate or nitrite. *Applied Microbiology and Biotechnology*. **68**: 272 – 282.

Hubert, C., and G. Voordouw. 2007. Oil field souring control by nitrate-reducing *Sulfurospirillum* spp. that outcompete sulfate-reducing bacteria for organic electron donors. *Applied and Environmental Microbiology*. **73** (8): 2644 – 2652.

Icgen, B. and S. Harrison. 2006. Identification of population dynamics in sulfate-reducing consortia on exposure to sulfate. *Research in Microbiology*. **157**: 922 – 927.

Ilhan-Sungur, E., N. Cansever and A. Cotuk. 2007. Microbial corrosion of galvanized steel by a freshwater strain of sulphate reducing bacteria (*Desulfovibrio* sp.). *Corrosion Science*. **49**: 1097 – 1109.

Ismail, Kh.M., A. Jayaraman, T.K. Wood and J.C. Earthman. 1999. The influence of bacteria on the passive film stability of 304 stainless steel. *Electrochimica Acta*. **44**: 4685 – 4692.

Ito, T., S. Okabe, H. Satoh and Y. Watanabe. 2002. Successional development of sulfate-reducing bacterial populations and their activities in a wastewater biofilm growing under microaerophilic conditions. *Applied and Environmental Microbiology*. **68** (3): 1392 – 1402.

Iverson, W.P. 1987. Microbial corrosion of metals. *Advances in Applied Microbiology*. **32**: 1 – 36.

Jack, T. R., E. Lee, and J. Mueller. 1985. Anaerobic gas production: controlling factors, p. 167–180. *In* J. E. Zajic and E. C. Donaldson (ed.), Microbes and oil recovery. Proceedings of the International Conference on Microbial Enhancement of Oil Recovery. Petroleum Bioresources, El Paso, Tex.

Jack, R.F., D.B. Ringelberg and D.C. White. 1992. Differential corrosion rates of carbon steel by combinations of *Bacillus* sp., *Hafnei alvei* and *Desulfovibrio gigas* established by phospholipid analysis of electrode biofilm. *Corrosion Science*. **33** (12): 1843 - 1853.

Jack, T.R., and D.W.S. Westlake. 1995. Control in industrial settings. Biotechnology handbooks. Vol. 8. Series editors: T. Atkinson and R.F. Sherwood. Sulfate-Reducing Bacteria. L.L. Barton. Plenum Press. New York. 265 – 292.

Jack, T.R. 2002. Biological Corrosion Failures. *ASM Handbook*. **11**: 881 – 890.

Jaen, J.A., J. Iglesias and C. Hernandez. 2012. Analysis of short-term steel corrosion products formed in tropical marine environments of Panama. *International Journal of Corrosion*. doi:10.1155/2012/162729.

Jain, D.K. 1995. Evaluation of the semisolid Postgate's B medium for enumerating sulfate-reducing bacteria. *Journal of Microbiological Methods*. **22**: 27 – 38.

Jan-Roblero, J., J.M. Romero, M. Amaya and S. Le Borgne. 2004. Phylogenetic characterization of a corrosive consortium isolated from a sour gas pipeline. *Applied Microbiology and Biotechnology*. **64**: 862 - 867.

Javaherdashti, R. 1999. A review of some characteristics of MIC caused by sulfate-reducing bacteria: past, present and future. *Anti-Corrosion Methods and Materials*. **46** (3): 173-180.

Jayaraman, A., E.T. Cheng, J.C. Earthman and T.K. Wood. (a). 1997. Axenic aerobic biofilms inhibit corrosion of SAE 1018 steel through oxygen depletion. *Applied Microbiology and Biotechnology*. **48**: 11-17.

Jayaraman, A., J.C. Earthman and T.K. Wood. (b). 1997. Corrosion inhibition by aerobic biofilms on SAE 1018 steel. *Applied Microbiology and Biotechnology*. **47**: 62 - 68.

Jayaraman, A., E.T. Cheng, J.C. Earthman and T.K. Wood. (c). 1997. Importance of biofilm formation for corrosion inhibition of SAE 1018 steel by axenic aerobic biofilms. *Journal of Industrial Microbiology and Biotechnology*. **18**: 396 – 401.

Jayaraman, A., A.K. Sun and T.K. Wood. 1998. Characterization of axenic *Pseudomonas fragi* and *Escherichia coli* biofilms that inhibit corrosion of SAE 1018 steel. *Journal of Applied Microbiology*. **84**: 485 – 492.

Jayaraman, A., D. Ornek, D.A. Duarte, C.-C. Lee, F.B. Mansfeld and T.K. Wood. (a). 1999. Axenic aerobic biofilms inhibit corrosion of copper and aluminium. *Applied Microbiology and Biotechnology*. **52**: 787 – 790.

Jayaraman, A., F.B. Mansfeld and T.K. Wood. (b). 1999. Inhibiting sulfate-reducing bacteria in biofilms by expressing the antimicrobial peptides indolicidin and bactenecin. *Journal of Industrial Microbiology and Biotechnology*. **22**: 167 – 175.

Jenneman, G. E., M. J. McInerney, and R. M. Knapp. 1986. Effect of nitrate on biogenic sulfide production. *Applied and Environmental Microbiology*. **51:**1205–1211.

Johansson L. –S., and T. Saastamoinen. 1999. Investigating early stages of biocorrosion with XPS: AIS 304 stainless steel exposed to Burkholderia species. *Applied Surface Science*. **144-145**: 244 – 248.

Johnsirani, V., J. Sathiyabama, S. Rajendran and A.S. Prabha. 2012. Inhibitory mechanism of carbon steel corrosion in seawater by an aqeous extract of henna leaves. *International Scholarly Research Network*. doi:10.5402/2012/574321.

Jones, D.A. 1995. Principles and prevention of corrosion. 2nd edition, Prentice Hall.

Kan, J., P. Chellamuthu, A. Obraztsova, J.E. Moore and K.H. Nealson. 2011. Diverse bacterial groups are associated with corrosive lesions at a Granite Mountain Record Vault (GMRV). *Journal of Applied Microbiology*. **111**: 329 - 337.

Kang, C.K., and Y.-S. Lee. 2011. The surface modification of stainless steel and the correlation between the surface properties and protein adsorption. *Journal of Material Science*. **18**: 1389 - 1398.

Kawai, M., E. Matsutera, H. Kanda, N. Yamaguchi, K. Tani and M. Nasu. 2002. 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and Denaturing Gradient Gel Electrophoresis. *Applied and Environmental Microbiology*. **68** (2): 699 - 704.

Kaye, J.Z, J.B. Sylvan, K.J. Edwards and J.A. Baross. 2011. *Halomonas* and *Marinobacter* ecotypes from hydrothermal vent, subseafloor, deep-sea environments. *FEMS Microbiology and Ecology*. **75**: 123 - 133.

Kebbouche-Gana, S., and M.L. Gana. 2012. Biocorrosion of carbon steel by nitrate-utilizing consortium of sulfate-reducing bacteria obtained from an Algerian oil field. *Annals of Microbiology*. **62**: 203 - 210.

Keevil, C. W., J. T. Walker, J. McEvoy, and J. S. Colbourne. 1989. Detection of biofilms associated with pitting corrosion of copper pipework in Scottish hospitals. Biocorrosion. Gaylarde, C. C.; Morton, L. H. G. (eds.), Biodeterioration society occasional publication No.5. Biodeterioration Society, Lancashire, U.K. 99-117.

Keresztes, Z., I. Felhosi and E. Kalman. 2001. Role of redox properties of biofilms in corrosion processes. *Electrochimica Acta*. **46**: 3841 – 3849.

Khan, S., F. Haq, F. Hasan, K. Saeed and Rahatullah. 2012. Isolation and characterization of acidophilic sulphur and iron oxidizing *Acidothiobacillus ferrooxidans* from Black Shale. *International Journal of Biosciences*. **2** (2): 85 – 94.

Kjeldsen, K.U., B.V. Kjellerup, K. Egli, B. Frolund, P.H. Nielsen and K. Ingvorsen. 2007. Phylogenetic and functional diversity of bacteria in biofilms from metal surfaces of an alkaline district heating system. *FEMS Microbiology Ecology*. **61**: 384 - 397.

Kjellerup, B.V., T.R. Thomsen, J.L. Nielsen, B.H. Olesen, B. Frolund and P.H. Nielsen. 2005. Microbial diversity in biofilms from corroding heating systems. *Biofouling*. **21** (1): 19 - 29.

Kjellerup, B.V., K.U. Kjeldsen, F. Lopes, L. Abildgaard, K. Ingvorsen, B. Frolund, K.R. Sowers and P.H. Nielsen. 2009. Biocorrosion and biofilm formation in a nutrient limited heating system subjected to alternating microaerophilic conditions. *Biofouling*. **25** (8): 727 - 737.

Klee, A.J. 1996. Most Probable Number Calculator version 4.04. *in*. U.S. Environmental Protection Agency, Risk Reduction Engineering Laboratory, Cincinnati, OH.

Kokare, C.R., S. Chakraborty, A.N. Khopade and K.R. Mahadik. 2009. Biofilm: Importance and Applications. *Indian Journal of Biotechnology*. **8**: 159 - 168.

Korenblum, E., I. Von der Weid, G.V. Sebastian, L. Seldin, M. Magalhaes de Paiva, F.C.M. Magalhaes and C.M.L.M. Coutinho. 2005. Inhibition of biofilm formation of *Bacillus pumilus* by antimicrobial substances produced by different oil reservoir *Bacillus* strains. 1-6.

Kumaraswamy, R., S. Ebert, M.R. Gray, P.M. Fedorak and J.M. Foght. 2010. Molecular- and cultivation-based analyses of microbial communities in oil field water and in microcosms amended with nitrate to control H_2S production. *Applied Microbiology and Biotechnology*. **89** (6): 2027 - 2242.

Laitinen, T. 2002. Localized corrosion of stainless steel in chloride, sulfate and thiosulfate containing environments. *Corrosion Science*. **42**: 421 – 441.

Landoulsi, J., K. El Kirat, C. Richard, D. Feron and S. Pulvin. 2008. Enzymatic approach in Microbial-Influenced Corrosion: A review based on stainless steels in natural waters. *Environmental Science and Technology*. **42** (7): 2233 – 2242.

Lane, R.A. 2005. Under the microscope: understanding, detecting, and preventing microbiologically influenced corrosion. The AMPTIAC Quarterly. **9** (1): 3- 8.

Larsen, J. 2002. Downhole nitrate applications to control sulfate reducing bacteria activity and reservoir souring. NACE - International Corrosion Conference Series, Denver, CO., Paper no. 02025.

Larsen, J., S. Zwolle, B.V. Kjellerup, B. Frolund, J.L. Nielsen and P.H. Nielsen. 2005. Identification of bacteria causing souring and biocorrosion in the Halfdan field by application of new molecular techniques. *NACE Proceedings*. Paper No. 05629.

Larsen, J., T.L. Skovhus, M. Agerbaek, T.R. Thomsen and P.H. Nielsen. 2006. Bacterial diversity study applying novel molecular methods on Halfdan produced waters. NACE - International Corrosion Conference Series, Houston, Paper 06668.

Lata, S., C. Sharma and A.K. Singh. 2012. Comparison of biocorrosion due to *Desulfovibrio desulfuricans* and *Desulfotomaculum nigrificans* bacteria. *Journal of Materials Engineering and Performance*. DOI:10.1007/S11665-012-0283-3.

Law, D.W., J. Cairns, S.G. Millard and J.H. Bungey. 2004. Measurement of loss of steel from reinforcing bars in concrete using linear polarization resistance measurements. *NDT&E International*. **37**: 381 – 388.

Lebedev, A.N. and A.S. Derbyshev. 1978. Kinetics of carbon steel corrosion in Caspian seawaters and chloride solutions. *Protection of Metals*. **14** (16): 575 – 577.

Leda, H.O., S. Simison and J.P. Busalmen. 2006. Stainless steels can be cathodically protected using energy stored at the marine sediment/seawater interface. *Environmental Science and Technology*. **40** (25): 6473 – 6478.

Lee, W. and W. G. Characklis. 1993. Corrosion of mild steel under anaerobic biofilm. *Corrosion.* **49:** 186-198.

Lee, A.K., M.G. Buehler, and D.K. Newman. 2006. Influence of a dual-species biofilm on the corrosion of mild steel. *Corrosion Science*. **48**: 165 – 178.

Lewandowski, Z., T.E. Cloete, S.C. Dexter, W.H. Dickinson, Y. Kikuchi, B. Little, F. Mansfeld, H. Rossmoore, W. Sand and H.A. Videla. 2003. MIC Issues: Commentary from the Corrosion 2002 MIC Panel Discussion. *NACE Corrosion, 2003*. 03560/1 – 03560/6.

Lewandowski, Z., and H. Beyenal. 2008. Mechanisms of Microbially Influenced Corrosion. *Springer Series on Biofilms*. doi:10.1007/7142: 35 - 64.

Li, W., B. Yuan, C. Wang, L. Li and S. Chen. 2012. Dynamic sensing of localized corrosion at the metal/solution interface. *Sensors (Basel)*. **12** (4): 4962 – 4973.

Lin, J. and R. Ballim. 2012. Biocorrosion control: Current strategies and promising alternatives. *African Journal of Biotechnology*. **11** (91): 15736-15747.

Little, B., P. Wagner and F. Mansfeld. 1991. Microbiologically Influenced Corrosion of metals and alloys. *International Materials Reviews*. **36** (6): 253 – 272.

Little, B. and R. Ray. 2002. A perspective on corrosion inhibition by biofilms. *Corrosion*. **58**: 424 – 428.

Little, B.J., J.S. Lee and R.I. Ray. 2006. Diagnosing Microbiologically Influenced Corrosion: A State-of-the Art Review. *Corrosion*. **62** (11): 1006 - 1017.

Little, B., R. Ray and J. Lee. 2011. Diagnosing, measuring and monitoring Microbiologically Influenced Corrosion (MIC). *Symposium on Microbiologically Influenced Corrosion*. 1 – 10.

Long, L., H. Zhu, Q. Yao and Y. Ai. 2008. Analysis of bacterial communities associated with spores of *Gigaspora margarita* and *Gigaspora rosea*. *Plant Soil*. **310**: 1 - 9.

Lopes, F.A., P. Morin, R. Oliveira and L.F. Melo. 2006. Interaction of *Desulfovibrio desulfuricans* biofilms with stainless steel surface and its impact on bacterial metabolism. *Journal of Applied Microbiology*. **101**: 1087 – 1095.

Lopez, E., A. Osella and L. Martino. 2006. Controlled experiments to study corrosion effects due to external varying fields in embedded pipelines. *Corrosion Science*. **48**: 389 – 403.

Lopez, M.A, F.J.Z. Serna, J. Jan-Roblero, J.M. Romero and C. Hernandez-Rodriguez. 2006. Phylogenetic analysis of a biofilm bacterial population in a water pipeline in the Gulf of Mexico. *FEMS Microbiology and Ecology*. **58**: 145 - 154.

Loto, C.A., O.A. Omotosha and A.P.I. Popoola. 2011. Inhibition effect of potassium dichromate on the corrosion protection of mild steel reinforcement in concrete. *International Journal of Physical Sciences*. **6** (9): 2275 - 2284.

Lowmunkhong, P., D. Ungthararak and P. Sutthivaiyakit. 2010. Tryptamine as a corrosion inhibitor of mild steel in hydrochloric acid solution. *Corrosion Science*. **52**: 30 – 36.

Lu, W.-K, R.L. Elsenbaumer and B. Wessling. 1995. Corrosion protection of mild steel by coatings containing polyaniline. *Synthetic metals*. **71**(1-3): 2163 – 2166.

Lutterbach, M.T.S., and F.P. de Franca. 1997. Biofilm formation on brass coupons exposed to cooling water. *Brazilian Journal of Chemical Engineering*. **14** (1): 81 - 87.

Lutey, R. W. 1992. Identification and detection of microbiologically influenced corrosion. Videla, H. A.; Lewandowski, Z.; Lutey, R. W. (eds.), Proceedings of NSF-CONICET Workshop Biocorrosion and Biofouling, Metal/Microbe Interactions, Mar del Plata, Argentina, Buckman Laboratories International, TN. 146-158.

Ma, Y., Y. Li and F. Wang. 2009. Corrosion of low carbon steel in atmospheric environments of different chloride content. *Corrosion Science*. **51**: 997 – 1006.

Macdonald, R., and V.S. Brozel. 2000. Community analysis of bacterial biofilms in a simulated recirculating cooling-water system by fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes. *Water Research*. **34** (9): 2439-2446.

Majumdar, I., F. D'Souza and N.B. Bhosle. 1999. Microbial exopolysaccharides: Effect on corrosion and partial chemical characterization. *Journal of Indian Institute of Science*. **79**: 539 – 550.

Malucknov, B.S. 2012. Corrosion of steels induced by microorganisms. *Metallurgical Material Engineers*. **18** (3): 223 – 231.

Manga, S.S., S.B. Oyeleke, A.D. Ibrahim, A.A. Aliero and A.I. Bagudo. 2012. Influence of bacteria associated with corrosion of metals. *Continental Journal of Microbiology*. **6** (1): 19-25.

Mansfeld, F. 2007. The interaction of bacteria and metal surfaces. *Electrochimica Acta*. **52**: 7670 – 7680.

Marchesi, J.R., T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiam and W.G. Wade. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*. **64** (2): 795 - 799.

Martiny, A.C., H.-J. Albrechtsen, E. Arvin and S. Molin. 2005. Identification of bacteria in biofilm and bulk water samples from a nonchlorinated model drinking water distribution system: detection of a large nitrite-oxidizing population associated with *Nitrospira* spp. *Applied and Environmental Microbiology*. **71** (12): 8611 – 8617.

Maruthamuthu, S., S. Mohanan, A. Rajeskar, N. Muthukumar, S. Ponmarippan, P. Subramanian and N. Palaniswamy. 2005. Role of corrosion inhibitors on bacterial corrosion in petroleum product pipeline. *Indian Journal of Chemical Technology*. **12**: 567 - 575.

Marques, J.M., F.P. de Almeida, U. Lins, L. Seldin and E. Koremblum. 2012. Nitrate treatment effects on bacterial community biofilm formed on carbon steel in produced water stirred tank bioreactor. *World Journal of Microbiology and Biotechnology*. **28**: 2355 – 2363.

Masuda, H. 2001. Effect of magnesium chloride liquid thickness on atmospheric corrosion of pure iron. *Corrosion*. **57** (2): 99 – 109.

Matsumura, Y., K. Yamada, M. Takahashi, Y. Kikuchi and T. Tsuchido. 2007. Properties of bacterial corrosion of stainless steel and its inhibition by protamine coating. *Biocontrol Science*. **12**: 21 - 29.

McCauley, A., C. Jones and J. Jacobsen. 2003. Soil pH and organic matter. *Nutrient Management-a self study course from the MSU extension service continuing education series*. **8**: 1 – 12.

McEwan, J. 2005. Corrosion Control in Southern Africa. University of Witwatersrand. (http://www.corrosioninstitute.org.za/)

McInerney, M. J., K. L. Sublette, V. K. Bhuparthiaraju, J. D. Coates, and R. M. Knapp. 1993. Causes and control of microbially induced souring, p.363–371. *In* E. T. Premuzic and A. Woodhead (ed.), Developments in petroleum science. 39. Microbial enhancement of oil recovery—recent advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. Elsevier, Amsterdam, The Netherlands.

McLeod, E.S., R. MacDonald and V.S. Brozel. 2002. Distribution of *Shewanella putrefaciens* and *Desulfovibrio vulgaris* in biosulfidogenic biofilms of industrial cooling water systems determined by fluorescent *in situ* hybridization. *Water SA*. **28**: 123 – 128.

McNeill, L.S., and M. Edwards. 2002. The importance of temperature in assessing iron pipe corrosion in water distribution systems. *Environmental Monitoring and Assessment.* **77** (3): 229 - 242.

Melchers, R.E. (a). 2003. Probabilistic models for corrosion in structural reliability assessment. Part 1: Empirical Models. *Journal of Offshore Mechanics and Artic Engineering*. **125** (4): 264 – 271.

Melchers, R.E. (b). 2003. Probabilistic models for corrosion in structural reliability assessment. Part 11: Models based on mechanics. *Journal of Offshore Mechanics and Artic Engineering*. **125** (4): 272 – 280.

Melchers, R.E. (c). 2003. Probabilistic Model for marine corrosion of steel for structural reliability assessment. *Journal of Structural Engineering*. **129** (11): 1484-1493.

Melchers, R.E. and R. Jeffrey. 2004. Influence of water velocity on marine immersion corrosion of mild steel. *Corrosion*. **60** (1): 84 – 94.

Melchers, R.E. and R. Jeffrey. 2005. Early corrosion of mild steel in seawater. *Corrosion Science*. **47** (7): 1678 – 1693.

Melchers, R.E. (a). 2006. Recent progress in the modeling of corrosion of structural steel immersed in seawaters. *Journal of Infrastructure Systems*. **12** (3): 154 – 162.

Melchers, R.E. (b). 2006. Pitting corrosion of mild steel under marine anaerobic conditions. Part 1: Experimental observations. *Corrosion*. **62**: 981 – 988.

Melchers, R.E. 2007. The effects of water pollution on the immersion corrosion of mild and low alloy steels. *Corrosion Science*. **49**: 3149 – 3167.

Melchers, R.E. 2008. A new interpretation of the corrosion loss processes for weathering steels in marine atmospheres. *Corrosion Science*. **50**: 3446 – 3454.

Melchers, R.E., and R. Jeffrey. 2008. The critical involvement of anaerobic bacterial activity in modeling the corrosion behaviour of mild steel in marine environments. *Electrochimica Acta*. **54**: 80-85.

Miranda, E., M. Bethencourt, F. J. Botana, M. J. Cano, J. M. Sa´nchez-Amaya, A. Corzo, J. Garcı´a de Lomas, M. L. Fardeau and B. Ollivier. 2006. Biocorrosion of carbon steel alloys by an hydrogenotrophic sulfate-reducing bacterium *Desulfovibrio capillatus* isolated from a Mexican oil field separator. *Corrosion Science*. **48**: 2417–2431.

Mirgane, S.R. and B.G. Ramdas. 2009. Investigation of soil corrosivity in Thane Region of Maharashtra, India. *Research Journal of Agriculture and Biological Sciences*. **5** (5): 680 – 688.

Mogawer, H., and R. Brown. 2011. The effect of calcium nitrite on the electrochemical characterization of 3003 aluminium alloys in seawater. *Journal of American Science*. **7** (5): 537 - 541.

Mohanty, S.S., T. Das, S.P. Mishra and G.R. Chaudhury. 2000. Kinetics of SO_4^{2-} reduction under different growth media of sulfate-reducing bacteria. bioMetals, Kluwer Publishers. 73 – 76.

Moller, H., E.T. Boshoff and H. Froneman. 2006. The corrosion behaviour of a low carbon steel in natural and synthetic seawaters. *The Journal of The South African Institute of Mining and Metallurgy*. **106**: 585 – 592.

Momba, M.N.B., R. Kfir, S.N. Venter and T.E. Cloete. 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. *Water SA*. **26** (1): 59 – 66.

Moradi, M., J. Duan, H. Ashassi-Sorkhabi and X. Luan. 2011. De-alloying of 316 stainless steel in the presence of a mixture of metal oxidizing bacteria. *Corrosion Science*. **53** (12): 4282 - 4290.

Morales, J., P. Esparza, S. Gonzalez, R. Salvarezza and M.P. Arevalo. 1993. *Corrosion Science*. **34** (9): 1531 - 1540.

Motamedi, M., and K. Pedersen. 1998. *Desulfovibrio aespoeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Aspo hard rock laboratory, Sweden. *International Journal of Systematic Bacteriology*. **48**: 311 – 315.

Mousavi, M., M. Mohammadalizadeh and A. Khosravan. 2011. Theoretical investigation of corrosion inhibition effect on imidazole and its derivatives on mild steel using cluster model. *Corrosion Science*. **53**: 3086 - 3091.

Mudryk, Z.J., B. Podgorska, and J. Bolalek. 2000. The occurrence and activity of sulphate-reducing bacteria in the bottom sediments of the Gulf of Gdansk. *Oceanologia*. **42** (1): 105 – 117.

Mueller, R.F. (1996). Microbial dynamics in souring oil reservoirs. PhD thesis, Montana State University in Bozeman.

Murga, R., T.S. Forster, E. Brown, J.M. Pruckler, B.S. Fields and R.M. Donlan. 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology*. **147** (11): 3121 - 3126.

Murray, A.E., J.T. Hollibough and C. Orrego. 1996. Phylogenetic compositions of bacterioplankton from two California Estuaries compared by Denaturing Gradient Gel Electrophoresis of 16S rDNA fragments. *Applied and Environmental Microbiology*. **62** (7): 2676 - 2680.

Muyzer, G., E.C.D. Waal and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*. **59** (3): 695 - 700.

Myers, C., and K. H. Nealson. 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science*. **240**: 1319 -1321.

Nakatsu, C.H. 2007. Soil microbial community analysis using Denaturing Gradient Gel Electrophoresis. *Soil Science Society American Journal*. **71** (2): 562 – 571.

Nemati, M., T.J. Mazutinec, G.E. Jenneman, and G. Voordouw. (a). 2001. Control of biogenic H_2S production with nitrite and molybdate. *Journal of Industrial Microbiology and Biotechnology*. **26**: 350 - 355.

Nemati, M., T.J. Mazutinec, G.E. Jenneman, and G. Voordouw. (b). 2001. Mechanistic study of microbial control of hydrogen sulfide production in oil reservoirs. *Biotechnology and Bioengineering*. **74** (5): 128 - 136.

Neria-Gonzalez, I., E.T. Wang, F. Ramirez, J.M. Romero and C. Hernandez-Rodriguez. 2006. Characterization of bacterial community associated to biofilms of corroded oil pipelines from the southeast of Mexico. *Anaerobe*. **12**: 122-133.

Newson, T. 2002. Stainless steel – A family of Medical Device Materials. Business Briefing: Medical Device Manufacturing and Technology. 1 – 3.

Nielsen, P.H., W. Lee, Z.L. Lewandowski, M. Morison and W.G. Characklis. 1993. Corrosion of mild steel in an alternating oxic and anoxic biofilm. *Biofouling*. **7**: 267 - 284.

Nikolaev, Y.A., and Plakunov, V.K. 2007. Biofilm – "City of Microbes" or an Analogue of Multicellular Organisms? *Microbiology.* **76** (2): 125 – 138.

Nishikata, A., Y. Ichihara, Y. Hayashi, T. Tsuru. 1997. Influence of electrolyte layer thickness and pH on the initial stage of the atmospheric corrosion of iron. *Journal of Electrochemical Society*. **144** (4): 1244 – 1252.

Nizhegorodov, S. Yu, S.A. Voloskov, V.A. Trusov, L.M. Kapulkina and T.A. Syur. 2008. Corrosion of steels due to the action of microorganisms. *Metal Science and Heat Treatment*. **50** (3-4): 191 - 195.

Norhazilan, M.N., Y. Nordin, K.S. Lim, R.O. Siti, A.R.A. Safuan and M.H. Norhamimi. 2012. Relationship between soil properties and corrosion of carbon steel. *Journal of Applied Sciences Research*. **8** (3): 1739-1747.

Obuekwe, C.O., D.W.S. Westlake, F.D. Cook and J.W. Costerton. (a). 1981. Surface changes in mild steel coupons from the action of corrosion-causing bacteria. *Applied and Environmental Microbiology*. **41** (3): 766-774.

Obuekwe, C. 0., D. W. S. Westlake, J. A. Plambeck and F. D.Cook. (b). 1981. Corrosion of mild steel in cultures of ferric ion reducing bacterium isolated from crude oil polarization characteristics. *Corrosion*. **37**: 461- 467.

Obuekwe, C., D. Westlake and J.A. Plambeck. 1987. *Canadian Journal of Microbiology*. **33**: 272.

Oguzie, E.E., I.B. Agochukwu and A.I. Onuchukwu. 2004. Monitoring the corrosion susceptibility of mild steel in varied soil textures by corrosion product count technique. *Materials Chemistry and Physics*. **84**: 1-6.

Oguzie, E.E. 2005. Corrosion inhibition of mild steel in hydrochloric acid solution by methylene blue dye. *Materials Letters*. **59**: 1076-1079.

Okabe, S., T. Itoh, H. Satoh and Y. Watanabe. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. *Applied and Environmental Microbiology*. **65** (11): 5107 – 5116.

Oladele, S.K., and H.K Okoro. 2011. Investigation of corrosion effect of mild steel on orange juice. *African Journal of Biotechnology*. **10** (16): 3152 - 3156.

Oliveira, V.M., P.F. Lopes-Oliveira, M.R.Z. Passarini, C.B.A. Menezes, W.R.C. Oliveira, A.J. Rocha and L.D. Sette. 2011. Molecular analysis of microbial diversity in corrosion samples from energy transmission towers. *Biofouling: The Journal of Bioadhesion and Biofilm Research*. **27** (4): 435 - 447.

Orfei, L.H., S. Simison and J.P. Busalmen. 2006. Stainless steels can be cathodically protected using energy stored at the marine sediment/seawater interface. *Environmental Science and Technology*. **40** (20): 6473-6478.

Ornek, D., A. Jayaraman, B.C. Syrett, C.H. Hsu, F.B. Mansfeld and T.K. Wood. 2002. Pitting corrosion inhibition of aluminium 2024 by *Bacillus* biofilms secreting polyaspartate or g-polyglutamate. *Applied Microbiology and Biotechnology*. **58**: 651 – 657.

O'Sullivan, L.A., G. Webster, J.C. Fry, R.J. Parkes and A.J. Weightman. 2008. Modified linker-PCR primers facilitate complete sequencing of DGGE DNA fragments. *Journal of Microbiological Methods*. **75**: 579 - 581.

Ostwald, C. and H.J. Grabke. 2004. Initial oxidation and chromium diffusion. I. Effects of surface working on 9 – 20% Cr steels. *Corrosion Science*. **46**: 1113 – 1127.

Pankhania, I.P., L.A. Gow and W.A. Hamilton. 1986. The effect of hydrogen on the growth of *Desulfovibrio vulgaris* (Hildenborough) on lactate. *Journal of General Microbiology*. **132**: 3349-3356a.

Park, J.-W., and D.E. Crowley. 2005. Normalization of soil DNA extraction for accurate quantification of target genes by real-time PCR and DGGE. *BioTechniques*. **38** (4): 579 - 586.

Paul, S. 2012. Modeling to study the effect of environmental parameters on corrosion of mild steel in seawater using neural network. *International Scholarly Research Network*. doi:10.5402/2012/487351.

Pederson, A. and M. Hermansson. 1989. The effects on metal corrosion by *Serratia marcescens* and a *Pseudomonas* sp. *Biofouling* **1**: 313 – 322.

Pederson, A. and M. Hermansson. 1991. Inhibition of corrosion by bacteria. *Biofouling* **3**: 1 – 11.

Perez, E.J., R. Cabrera-Sierra, I. Gonzalez and F. Ramirez-Vives. 2007. Influence of *Desulfovibrio* sp. biofilm on SAE 1018 carbon steel corrosion in synthetic marine medium. *Corrosion Science*. **49**: 3580 – 3597.

Philips, S., H.J. Laanbroek and W. Verstraete. 2002. Origin, causes and effects of increased nitrite concentrations in aquatic environments. *Reviews in Environmental Science and Biotechnology*. **1**: 115 – 141.

Pope, D. H., D. J. Duquette, A. H. Johannes, and P. C. Wayner. 1984. Microbially influenced corrosion of industrial alloys. *Materials Performance*. **23**: 14-18.

Pope, D.H. and R.M. Pope. 1998. Guide for the monitoring and treatment of microbiologically influenced corrosion in the natural gas industry. GRI Report GRI-96/0488. Gas Research Institute. Des Plaines.

Potekhina, J.S., N.G. Sherisheva, L.P. Poretkina, A.P. Pospelov, T.A. Rakitina, F. Warnecke and G. Gottschalk. 1999. Role of microorganisms in corrosion inhibition of metals in aquatic habitats. *Journal of Applied Microbiology and Biotechnology*. **52** (5): 639 – 646.

Puyate, Y.T., and A. Rim-Rukeh. 2008. Some physico-chemical and biological characteristics of soil and water samples of part of the Niger Delta area, Nigeria. *Journal of Applied Science and Environmental Management.* **12** (2): 135 – 141.

Rainha, V. L. and I. T. E. Fonseca. 1997. Kinetics studies on the SRB influenced corrosion of steel: a first approach. *Corrosion Science*. **39**: 807-813.

Rajasekar, A., S. Maruthamuthu, N. Muthukumar, S. Mohanan, P. Subramanian and N. Palaniswamy. 2005. Bacterial degradation of naphta and its influence of corrosion. *Corrosion Science*. **47**: 257 – 271.

Rajasekar, A., T.G. Babu, S.K. Pandian, S. Maruthamuthu, N. Palaniswamy and A. Rajendran. (a). 2007. Biodegradation and corrosion behavior of manganese oxidizer *Bacillus cereus* ACE4 in diesel transporting pipeline. *Corrosion Science*. **49**: 2694 - 2710.

Rajasekar, A., S. Maruthamuthu, N. Palaniswamy and A. Rajendran. (b). 2007. Biodegradation of corrosion inhibitors and their influence on petroleum product pipeline. *Microbiological Research*. **162**: 355 - 368.

Rajasekar, A., B. Anandkumar, S. Maruthamuthu, Y.-P. Ting, P.K.S.M Rahman. 2010. Characterization of corrosive bacterial consortia isolated from petroleum-product-transporting pipelines. *Applied Microbiology and Biotechnology*. **85**: 1175 – 1188.

Rajasekar, A., and Y.-P. Ting. 2011. Role of inorganic and organic medium in the corrosion behaviour of *Bacillus megaterium* and *Pseudomonas* sp. in stainless steel SS 304. *Industrial and Engineering Chemistry Research*. **50**: 12534 - 12541.

Ramesh, S., and S. Rajeswari. 2004. Corrosion inhibition of mild steel in neutral aqueous solution by new triazole derivatives. *Electrochimica Acta*. **49**: 811-820.

Raskin, L., B.E. Rittmann and D.A. Stahl. 1996. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Applied and Environmental Microbiology*. **62**: 3847 – 3857.

Reinsel, M. A., J. T. Sears, P. S. Stewart, and M. J. McInerney. 1996. Control of microbial souring by nitrate, nitrite or glutaraldehyde injection in a sandstone column. Journal of Industrial Microbiology. **17:**128–136.

Rempel, C.L., R.W. Evitts and M. Nemati. 2006. Dynamics of corrosion rates associated with nitrite or nitrate mediated control of souring under biological conditions simulating an oil reservoir. *Journal of Industrial Microbiology and Biotechnology*. **33** (10): 878 - 886.

Rice, A., M. Hamilton and A. Camper. 2000. Apparent surface associated lag time in growth of primary film cells. *Microbial Ecology*. **40**: 8 - 15.

Rogers, S.W., T.B. Moorman and S.K. Ong. 2007. Fluorescent in situ hybridization and microautoradiography applied to ecophysiology in soil. *Soil Science Society of America Journal*. **71** (2): 620-631.

Rosliza, R., W.B. Wan Nik, and H.B. Senin. 2008. The effect of inhibitor on the corrosion of aluminum alloys in acidic solutions. *Materials Chemistry and Physics*. **107**: 281 – 288.

Rothwell, G.P. 1979. Corrosion phenomena – An introduction. (http://www.npl.co.uk/lmm/docs/introduction to corrosion phenomena.pdf).

Rubio, C., C. Ott, C. Amiel, I. Dupont-Moral, J. Travert and L. Mariey. 2006. Sulfato/thiosulfato reducing bacteria characterization by FT-IR spectroscopy: A new approach to biocorrosion control. *Journal of Microbiological Methods*. **64** (3): 287 – 296.

Ryhl-Svendsen, M. 2008. Corrosivity measurements of indoor museum environments using lead coupons as dosimeters. *Journal of Cultural Heritage*. **9**: 285 - 293.

Sambrook, J., E.F. Fristsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanchez, O., J.M. Gasol, R. Massana, J. Mas and C. Pedros-Alio. 2007. Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. *Applied and Environmental Microbiology*. **73** (18): 5962 - 5967.

Sand, W. 1997. Microbial mechanisms of deterioration of inorganic substrates – a general mechanistic overview. *International Biodeterioration and Biodegradation*. **40** (2-4): 183 – 190.

Sandbeck, K. A., and D. O. Hitzman. 1995. Biocompetitive exclusion technology: a field system to control reservoir souring and increase production, p. 311–320. *In* R. Byrant and K. L. Sublette (ed.), Proceedings of the 5th International Conference on Microbial Enhanced Oil Recovery and Related

Biotechnology for Solving Environmental Problems. National Technical and Information Services, Springfield, Va.

Sanders, P.F. 1988. Control of biocorrosion using laboratory and field assessments. *International Biodeterioration.* **24**: 239 – 246.

Sanders, P.F. 2003. Novel methods for controlling microbial problems without using bactericides. *Saudi Aramco Journal of Technology*. 2 – 14.

Santana, J.J., F.J. Santana, J.E. Gonzalez, R.M. Souto, S. Gonzalez and J. Morales. 2012. Electrochemical analysis of the microbiologically influenced corrosion of AISI 304 stainless steel by sulphate reducing bacteria associated with *Bacillus cereus*. *International Journal of Electrochemical Science*. **7**: 711-724.

Santegoeds, C.M., T.G. Ferdelman, G. Muyzer and D. DE Beer. 1998. Structural and functional dynamics of sulfate-reducing populations in bacterial biofilms. *Applied and Environmental Microbiology*. **64** (10): 3731 - 3739.

Sarro, M.I., A.M. Garcia and D.A. Moreno. 2005. Biofilm formation in spent nuclear fuel pools and bioremediation of radioactive water. *International Microbiology*. **8**: 223 - 230.

Sass, H., J. Steuber, M. Kroder, P.M. Kroneck and H. Cypionka. 1992. Formation of thionates by freshwater and marine strains of sulphate-reducing bacteria. *Archives of Microbiology*. **158**: 418 – 421.

Satheesh, S. and S.G. Wesley. 2010. Biofilm development on acrylic coupons during the initial 24 hour period of submersion in a tropical coastal environment. *International Journal of Oceanography and Hydrobiology*. **39** (1): 27 - 38.

Seth, A.D., and R.G.J. Edyvean. 2006. The function of sulfate-reducing bacteria in corrosion of potable water mains. *International Biodeterioration and Biodegradation*. **58**: 108 – 111.

Sette, L.D., K.C. Simioni, S.P. Vasconcellos, L.J. Dussan, E.V. Neto and V.M. Oliveira. 2007. Analysis of the composition of bacterial communities in oil reservoirs from a southern offshore Brazilian basin. Antonie Leeuwenhoek. **91**: 253 - 266.

Schiermeyer, E.M., P.P. Provencio and D.E. Northup. 2000. Microbially induced iron oxidation: What, where, how. (http://www.osti.gov/)

Schwermer, C. U., G. Lavik, R. M. M. Abed, B. Dunsmore, T. G. Ferdelman, P. Stoodley, A. Gieseke and D. de Beer. 2008. Impact of nitrate on the structure and function of bacterial biofilm communities in pipelines used for injection of seawater into oil fields. *Applied and Environmental Microbiology*. **74** (9): 2841–2851.

Scott, P.J.B. 2004. Part 1. Expert Consensus on MIC: Prevention and Monitoring. *Materials Performance*. **43** (3): 1 – 6.

Sheng, X., Y.P. Ting and S.O. Pehkonen. 2007. Force measurements of bacterial adhesion on metals using a probe atomic force microscope. *Journal of Colloid and Interface Science*. **310**: 661 – 669.

Sheng, X., S.O. Pehkonen and Y.-P. Ting. 2012. Biocorrosion of stainless steel 316 in seawater: inhibition using an azole type derivative. *Corrosion Engineering, Science and Technology*. **47** (5): 388 – 393.

Sherar, B.W.A., P.G.Keech and D.W.Shoesmith. 2011. Carbon steel corrosion under anaerobic-aerobic cycling conditions in near-neutral pH saline solutions - Part 1: long term corrosion behaviour. *Corrosion Science,* doi: 10.1016/j/corsci.2011.07.015

Shreir, L. L. 1963. The microbiology of corrosion. *Corrosion,* 1, Wiley, *J* . , New York 252-264.

Silva, A.M.A., T.M. Santiago, C.R. Alves, M.I.F. Guedes, J.A.K. Freire, R.H.S.F. Vieira and R.C.B. da Silva. 2007. An evaluation of the corrosion behavior of aluminium surfaces in the presence of fungi using atomic force microscopy and other tests. *Anti-Corrosion Methods and Materials*. **54** (5): 289 – 293.

Simoes, M., L.C. Simoes and M.J. Vieira. 2010. A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*. **43**: 573 - 583.

Singer, E., E.A. Webb, W.C. Nelson, J.F. Heidelberg, N. Ivanova, A. Pati and K.J. Edwards. 2011. Genomic potential of *Marinobacter aquaeolei*, a biogeochemical "Opportunitroph". *Applied and Environmental Microbiology*. **77** (8): 2763 - 2771.

Singh, D.D.N., S. Yadav and J.K. Saha. 2008. Role of climatic conditions on corrosion characteristics of structural steels. *Corrosion Science*. **50**: 93 – 110.

Singh, A., C. Sharma and S. Lata. 2011. Microbial influenced corrosion due to *Desulfovibrio desulfuricans*. *Anti-Corrosion Methods and Materials*. **58** (6): 315 – 322.

Soracco, R. J., D. H. Pope, J. M. Eggers, and T. N. Effinger. 1988. Microbiologically influenced corrosion investigations in electric power generating stations. Proceedings of
NACE Corrosion 88, NACE, Houston, TX, Paper No. 83.

Sreekumari, K.R., K. Hirotani and Y. Kikuchi. 2004. Microbiologically influenced corrosion failure of AISI type 304 stainless steel in a wastewater treatment system. In: Proceedings of NACE Corrosion 2004, New Orleans, Paper, No. 04600.

Staley, J.T. and A. Konopka. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Reviews in Microbiology*. **39**: 321 - 346.

Starosvesky, D., 0. Khaselev, J. Starosvesky, R. Armon and J. Yahalom. 2000. Effect of iron exposure in SRB media on pitting initiation. *Corrosion Science*. **42:** 345-359.

Starosvetsky, J., D. Starosvetsky and R. Armon. 2007. Identification of microbiologically influenced corrosion (MIC) in industrial equipment failures. *Engineering Failure Analysis*. **14**: 1500 – 1511.

Starosvesky, J., D. Starosvetsky, B. Pokroy and R. Armon. 2008. Electrochemical behaviour of stainless steels in media containing iron-oxidizing bacteria (IOB) by corrosion process modeling. *Corrosion Science*. 50: 540-547.

Sturman, P.J. and D.M. Goeres. 1999. Control of hydrogen sulfide in oil and gas wells with nitrite injection. SPE paper 56772, SPE Annual Conference, Houston, TX.

Syed, S. 2006. Atmospheric corrosion of materials. *Emirates Journal of Engineering Research*. **11** (1): 1 – 24.

Tabari, K., M. Tabari and O. Tabari. 2011. Application of Biocompetitive Exclusion in prevention and controlling Biogenetic H_2S of petroleum reservoirs. *Australian Journal of Basic and Applied Sciences*. **5** (12): 715 – 718.

Tabatabaei, M., M.R. Zakaria, R.A. Rahim, A.-D.G. Wright, Y. Shirai, N. Abdullah, K. Sakai, S. Ikeno, M. Mori, N. Kazunori, A. Sulaiman and M.A. Hassan. 2009. PCR-based DGGE and FISH analysis of methanogens in an anaerobic closed digester tank for treating palm oil mill effluent. *Electronic Journal of Biotechnology*. **12** (3): 1 -12.

Tadros, H.R.Z, and M.G.M Osman. 2012. Relation between some physical and chemical parameters affecting on the corrosion rate of steel from Abu-Qir Till Al-Arish, Egypt during Winter 2008. *World Applied Sciences Journal*. **18** (3): 385 - 395.

Tamura K, J. Dudley, M. Nei and S. Kumar. 2007. *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution.* **24:**1596-1599.

Tanji, Y., Y. Morono, A. Soejima, K. Hori and H. Unno. 1999. Structural analysis of a biofilm which enhances carbon steel corrosion in nutritionally poor aquatic environments. *Journal of Bioscience and Bioengineering*. **88** (5): 551 – 556.

Tatnall, R. 1981. Fundamentals of bacterial induced corrosion. *Materials Performance.* **20**: 32-38.

Tavares, S.S.M., J.M. Pardal, L.D. Lima, I.N. Bastos, A.M. Nascimento and J.A. de Souza. 2007. Characterization of microstructure, chemical composition, corrosion resistance and toughness of a multipass weld joint of superduplex stainless steel UNS S32750. *Materials Characterization*. **58**: 610 – 616.

Teske, A., C. Wawer, G. Muyzer and N.B. Ramsing. 1996. Distribution of Sulfate-Reducing Bacteria in a stratified Fjord (Mariager Fjord, Denmark) as evaluated by most-probable number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology*. **62** (4): 1405 - 1415.

Thorstenson, T., G. Bodtker, B.L. Lillebo, T. Torsvik, E. Sunde, and J. Beeder. 2002. Biocide replacement by nitrate in seawater injection systems. Paper no. 02033,Proceedings of NACE Corrosion 2002, Denver, CO.

Valencia-Cantero, E., and J.J. Pena-Cabriales. 2003. The corrosion effects of sulfate- and ferric-reducing bacterial consortia on steel. *Geomicrobiology Journal*. **20**: 157 - 169.

Videla, H.A. 2002. Prevention and control of biocorrosion. *International Biodeterioration and Biodegradation*. **49**: 259 – 270.

Videla, H.A. and W.G. Characklis. 1992. Biofouling and microbially influenced corrosion. *International Biodegradation and Biodeterioration*. **29**: 195 – 207.

Videla, H.A. and L.K. Herrera. 2005. Microbiologically influenced corrosion: looking to the future. *International Microbiology*. **8**: 169 – 180.

Videla, H.A. and L.K. Herrera. 2007. Biocorrosion of oil recovery systems. Prevention and protection. An update. *Revista Tecnica de la Facultad de Ingenieria Universidad del Zulia*. **30**: 272 – 279.

Visscher, P.T., R.A. Prins and H. Gemerden. 1992. Rates of sulphate reduction and thiosulfate consumption in marine microbial mat. *FEMS Microbiology Ecology*. **86**: 283 – 294.

Voordouw, G., M. Nemati, and G.E. Jenneman. 2002. Use of nitrate-reducing, sulfide oxidizing bacteria to reduce souring in oil fields: interactions with SRB and effects on corrosion. Paper No. 02034. Proceedings of NACE Corrosion 2002, Denver, CO.

Wagner, M., R. Amann, H. Lemmer and K.-H. Schleifer. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology*. **59**: 1520 – 1525.

Wahid, A., D.L. Olson and D.K. Matlock. 1993. Corrosion of weldments. *ASM Handbook* **6**: 1065 – 1069. D.L. Olson, T.A. Siewert, S. Liu and G.R. Edwards, editors.

Weissenrieder, J. and C. Leygraf. 2004. *In Situ* studies of filiform corrosion of iron. *Journal of Electrochemical Society*. **151** (3): B165 – B171.

Wen, Z., and S. Hong-Bo. 2011. Applications of molecular biology and biotechnology in oil field microbial biodiversity research. *African Journal of Microbiology Research*. **5** (20): 3103 – 3112.

Westrich, J.T. and B.A. Berner. 1998. The effect of temperature on rates of sulfate reduction in marine sediments. *Geomicrobiology Journal*. **6**: 99 – 117.

Wilkinson, L. 1988. Systat: The system for statistics. *Evanston Systat*.

Xiuheng, W., Z. Kun, R. Nanqi, L. Nan and R. Lijiao. 2009. Monitoring microbial community structure and succession of an A/O SBR during start-up period using PCR-DGGE. *Journal of Environmental Sciences*. **21**: 223 - 228.

Xu, L-C., K-Y, Chan and H.H.P. Fang. 2002. Application of atomic force microscopy in the study of microbiologically influenced corrosion. *Materials Characterization*. **48**: 195 – 203.

Xu, C., Y. Zhang, G. Cheng and W. Zhu. 2007. Localized corrosion behaviour of 316L stainless steel in the presence of sulfate-reducing and iron-oxidizing bacteria. *Materials Science and Engineering*. A **443**:235 - 241.

Xu, C., Y. Zhang, G. Cheng and W. Zhu. 2008. Pitting corrosion behaviour of 316L stainless steel in the media of sulphate-reducing and iron-oxidising bacteria. *Materials Characterization*. **59**: 245 – 255.

Xu, P., Z. Xu, J. Wang, Y. Zhang and L. Zhang. 2012. MIC in circulating cooling water system. *Journal of Water Resource and Protection*. **4**: 203 - 206.

Yakimov, M.M., R. Denaro and M. Genovese. 2005. Natural microbial diversity in supercial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons. *Environmental Microbiology*. **7**: 1426 - 1441.

Yakimov, M.M., K.N. Timmis and P.N. Golyshin. 2007. Obligate oil-degrading marine bacteria. *Current Opinion in Biotechnology*. **18**: 257 - 266.

Ye, W., X. Liu, S. Lin, J. Tan, J. Pan, D. Li and H. Yang. 2009. The vertical distribution of bacterial and archael communities in the water and sediment of Lake Taihu. FEMS Microbiology and Ecology. **70** (2): 263 - 276.

Yuan, S.J., A.M.F. Choong and S.O. Pehkonen. 2007. The influence of the marine aerobic *Pseudomonas* strain on the corrosion of 70/30 Cu-Ni alloy. *Corrosion Science*. **49**: 4352 - 4385.

Zakaria, A.E., H.M. Gebreil and N.M. Abdelaal. 2012. Control of Microbiologically Induced Corrosion in petroleum industry using various preventive strategies. *Arab Journal of Nuclear Sciences and Applications*. **45** (2): 460 – 478.

Zhao, X., P. Wang, G. Lan, P. Shi, X. Chen and J. Wang. 2012. Study on the identification and biocorrosion behavior of one anaerobic thermophilic bacterium isolated from Dagang oil field. *African Journal of Microbiology*. **6** (32): 6218 – 6223.

Zhu, X.Y., J. Lubeck and J.J. Kilbane. 2003. Characterization of microbial communities in gas industry pipelines. *Applied and Environmental Microbiology*. **69**: 5354 - 5363.

Zhu, Q., H.T. Zhu, A.K. Tieu and C. Kong. 2011. Three dimensional microstructure study of oxide scale formed on a high-speed steel by means of SEM, FIB and TEM. *Corrosion Science*, doi: 10.1016/j.corsci.2011.07.004

Zinkevich, V., I. Bogdarina, H. Kang, M.A.W. Hill, R. Tapper and I.B. Beech. 1996. Characterisation of exopolymers produced by different isolates of marine sulphate-reducing bacteria. *International Biodeterioration and Biodegradation*. **37** (3-4): 163 – 172.

Zou, Y., J. Wang and Y.Y. Zheng. 2011. Electrochemical techniques for determining corrosion rate of rusted steel in seawater. *Corrosion Science*. **53**: 208 – 216.

Zuo, R., D. Ornek, B.C. Syrett, R.M. Green, C.H. Hsu, F.B. Mansfeld and T.K. Wood. 2004. Inhibiting mild steel corrosion from sulfate-reducing bacteria using antimicrobial-producing biofilms in Three-Mile-Island process water. *Applied Microbiology and Biotechnology*. **64**: 274 – 283.

Zuo, R., D. Ornek and T.K. Wood. 2005. Aluminium and mild steel binding peptides from phage display. *Applied Microbiology and Biotechnology*. **68**: 505 – 509.

Zuo, R. 2007. Biofilms: strategies for metal corrosion inhibition employing microorganisms. *Applied Microbiology and Biotechnology*. **76**: 1245 – 1253.