Influence of carbon steel grade on the initial attachment of bacteria and microbiologically influenced corrosion

M.A. Javeda,b, W.C. Neilc, P.R. Stoddarta,b and S.A. Wadea,b
afaculty of Science, Engineering and Technology, Swinburne university of Technology, Hawthorn, Victoria, Australia; bDefence Materials Technology Centre (DMTC), Melbourne, Australia; cDefence Science and Technology Group, Melbourne, Australia

ABSTRACT
The influence of the composition and microstructure of different carbon steel grades on the initial attachment (≤ 60 min) of Escherichia coli and subsequent longer term (28 days) corrosion was investigated. The initial bacterial attachment increased with time on all grades of carbon steel. However, the rate and magnitude of bacterial attachment varied on the different steel grades and was significantly less on the steels with a higher pearlite phase content. The observed variations in the number of bacterial cells attached across different steel grades were significantly reduced by applying a fixed potential to the steel samples. Longer term immersion studies showed similar levels of biofilm formation on the surface of the different grades of carbon steel. The measured corrosion rates were significantly higher in biotic conditions compared to abiotic conditions and were found to be positively correlated with the pearlite phase content of the different grades of carbon steel coupons.

KEYWORDS
Bacterial attachment; biofilm; carbon steel; microbiologically influenced corrosion; microstructure

Introduction

It is well documented that bacterial attachment and subsequent biofilm formation can influence corrosion reactions at the metal/solution interface (Little & Lee 2007; Javed et al. 2014). Biofilms form naturally on metal surfaces when they come into contact with an aqueous solution containing microorganisms. The development of a biofilm may cause a range of localised changes at the metal/solution interface, which can become quite different from the bulk environment (Borenstein 1994; Beech & Sunner 2004; Coetser & Cloete 2005). The resulting variations within and outside the biofilm can influence the corrosion process by changing electrochemical reactions at the metal/solution interface, the consequence of which is known as microbiologically influenced corrosion (MIC).

The corrosion of a metal or alloy can be influenced by a range of metallurgical variables including chemical composition, surface roughness, microstructure, grain boundaries, and residual stresses (Noel 2003). These metallurgical features are also considered to play an important role in bacterial attachment and the resulting MIC (Ibars et al. 1992; Walsh et al. 1993; Borenstein 1994; Sreekumari et al. 2004; Javed, Stoddart, McArthur et al. 2013).

Historically, a large focus of MIC research has been on the initiation and propagation of MIC in stainless steel (SS) alloys. One of the goals of those studies was to determine the role of metallurgical factors, including composition and microstructure, in the MIC of SS alloys. Biezma (1999) suggested that pitting attack at SS welds might be caused by the complexity of the microstructure, surface roughness and residual stresses. Subsequently, George et al. (2003) showed that the depletion of chromium from weld areas enhanced bacterial attachment and corrosion of the substratum near the welds. Studies have also shown that small grain size materials are more susceptible to bacterial attachment and subsequent MIC (Sreekumari et al. 2001; Almahamedh et al. 2011; Javed, Stoddart, McArthur et al. 2013). The presence of different microstructural phases in SSs is also expected to play an important role in MIC. However, there are conflicting reports about the preferential attack on the ferrite phase, the austenite phase or the interface between them (Borenstein 1988; de Damborenea et al. 2007). Stein (1991) concluded that either the austenite or ferrite phase can undergo preferential attack, depending on the oxidising or reducing nature of the environmental conditions.
In comparison to SS only a small number of studies have investigated the effect of metallurgical features on bacterial attachment and the subsequent MIC of carbon steel (CS). Mara and Williams (1972) studied the effect of microstructure on the MIC of cast iron and five different steel grades in the presence of sulphate reducing bacteria. They showed that the MIC rate increased with the carbon content of the steel. However, no explanation for the increase in the microbial corrosion rate with carbon content was provided. In another study, the corrosion of six different iron–carbon alloys was investigated in the presence of the bacterium Escherichia coli in anaerobic conditions (Ashton et al. 1973). While the bacterium was found to increase corrosion rates, no correlation was found between the carbon content and corrosion in this case. No work was undertaken in either of the two aforementioned reports on the effect of the carbon content on the initial bacterial attachment, which is a key step in subsequent biofilm formation and possibly resulting MIC. The influence of chemical composition and/or microstructure on MIC appears to be a complex issue and further studies are required to help elucidate their role in the MIC of different grades of carbon steel.

The majority of reports on the MIC of CS have studied longer term bacterial attachment (ie after biofilm formation) and the subsequent corrosion. The formation of a biofilm starts with the initial attachment of bacteria on a metal surface, and therefore it is important to understand the mechanisms that govern the early stages of bacterial attachment. Studies have shown that substratum surface roughness, even down to the nanometre range, can influence initial bacterial attachment and subsequent biofilm formation (Medilanski et al. 2002; Singh et al. 2011; Crawford et al. 2012). Therefore, it is important to include surface roughness as a variable when investigating the influence of metal microstructure on initial bacterial attachment. However, most previous studies of the relationship between substratum microstructure and bacterial attachment have not included any analysis of the roughness of the polished surfaces.

In addition to metal microstructure and surface roughness, the electrochemical potentials of metal substrata have also been found to influence the initial attachment of bacteria and biofilm formation. Studies have shown that bacteria can attach preferentially to anodic sites on a metal surface (Little et al. 1996, 1999; Franklin et al. 2000), which could be one of the reasons for preferential attachment of bacteria on grain boundaries (Shoesmith 1992; Noel 2003; Javed, Stoddart, McArthur et al. 2013). It has been suggested that the attraction of bacteria towards these anodic sites may be related to chemotaxis and/or surface charge effects. There are also studies which have explored the effect of applied surface potential/current on the number of bacterial cells attached (Gordon et al. 1981; Duan & Lin 2011; Nithila et al. 2012). Armon et al. (2001) studied the effect of electrochemical polarisation on bacterial attachment on six different metals. They found that the maximum attachment occurred in the potential range of −0.5 to 0.5 V (saturated calomel electrode) for all metals tested. They also found that greater bacterial attachment occurred onto metal surfaces with open circuit potential (OCP) closer to 0.0 V.

The present study was performed in order to explore the influence of chemical composition and microstructure of different grades of CS on initial bacterial attachment and MIC. At present there is limited understanding of whether the chemical composition and microstructural phases of different steel grades affect initial bacterial attachment and the resulting MIC. This study investigated the very early stages of bacterial attachment (≤ 60 min) and its correlation (if any) with the subsequent corrosion of different grades of CS in the presence of the bacterium E. coli. The work included a detailed analysis of the surface roughness parameters and microstructures of the different grades of CS. The effect of an externally applied potential on the initial attachment of bacterial cells on the different grades of CS was also studied. A range of surface characterisation techniques, including 3-D optical profilometry, optical microscopy and scanning electron microscopy (SEM) were used to evaluate the initial bacterial attachment, its correlation with biofilm formation and the resulting corrosion after a 28 day immersion period.

Materials and methods

Metal coupon preparation

Four different grades of CS, viz. 1010, 1020, 1030 and 1045, were used in this work. Their chemical compositions are shown in Table 1. The chemical composition was determined by the inductive coupled plasma atomic emission spectroscopy (ICP-AES) technique. The 1010 CS coupons were cut from a plate with dimensions of 25 mm × 25 mm × 3 mm, while the 1020, 1030 and 1045 coupons were prepared by cutting 25 mm diameter rods into 3 mm thick discs. The cutting was done using an automatic abrasive wheel cutting machine with water cooling (Struers Secotom-50, Brisbane, Queensland, Australia). A 2 mm diameter hole was drilled in each coupon, close to an edge, to allow them to be suspended vertically during long term immersion studies.

All of the coupons were polished using an automatic grinding and polishing machine (Struers Tegramin-25). The coupons were first ground through a series of silicon carbide papers with decreasing grit size (180, 240, 320, 600, 800, 1,200). Finally, the coupons were polished
through a sequence of fine polishing with 9 and 3 μm diamond-based suspensions to a 0.04 μm finish using silica suspension (Struers OP-U). After the final polishing step the coupons were marked with an indenter using a microhardness testing machine (Vickers hardness tester BUEHLER 1,600–6,806, Lake Bluff, IL, USA). The marking was used to create reference points for overlaying bacterial attachment images with microstructural data as discussed in Javed, Stoddart, McArthur et al. (2013) and Javed, McArthur, Stoddart et al. (2013). After marking, the coupons were ultrasonically cleaned with acetone for 10–15 min, rinsed with distilled water followed by ethanol, and then dried under warm air.

**Surface roughness measurement**

The surface roughness of the polished coupons was characterised using a 3-D optical profilometer (Bruker Contour GT-K1, Mannheim, Germany). The coupons were first scanned to take an overview of surface features and then topographical measurements were taken at 10 different locations on each coupon. Surface roughness measurements have been shown to be sensitive to the size of the scanned area (Truong et al. 2010); therefore measurements were performed for different scanning areas. The raw data obtained from the profilometer was filtered and processed according to the ISO 4288:1996 (ISO 1996) standard using VISION 64 software (v5.30, Bruker). Following the standard, surface roughness values were calculated after removing the waviness component of the scanned profiles using a suitable cut-off wavelength depending on the sampling length used.

**Microstructure analysis**

To observe the microstructure, the polished metal coupons were etched using 2% Nital, prepared by mixing 98 ml of absolute ethanol (100%) and 2 ml of nitric acid (70%). The microstructure of the etched metal coupons was recorded using an optical microscope with a 20 × objective lens. Examples of the microstructure of the different grades of CS are shown in Figure 1. Microstructural features including grain size, average grain diameter and percentage of pearlite phase were calculated from the images obtained. The steel coupons tested were used as received from the supplier. Therefore the final grain sizes will be affected by the temperatures and processing conditions to which the material was exposed during manufacturing. The grain size was measured using the circular intercept method as per the ASTM standard E-112 (ASTM 2011a). The percentage of pearlite phase was determined according to the ASTM standard as the small pearlite grain size made it difficult to distinguish between the pearlite phase and grain boundaries (see Figure 1a). Therefore, the percentage of pearlite phase in the 1010 CS was calculated as per the ‘lever-rule’ using an iron-carbon phase diagram (Campbell 2012).

**Preparation of test medium and bacterial culture**

All bacterial attachment tests were conducted using an M9 medium which contained essential salts and glucose as the energy source for bacterial growth. The M9 medium consisted of Na₂HPO₄ (6.816 g l⁻¹), K₂HPO₄ (3.83 g l⁻¹), NH₄Cl (1.07 g l⁻¹), NaCl (0.50 g l⁻¹), CaCl₂·2H₂O (0.0147 g l⁻¹), MgSO₄ 0.1 (0.1 g l⁻¹) and glucose (4 g l⁻¹). The medium was prepared by first mixing the salts in 1 l of MilliQ water. The pH of the medium was adjusted with HCl (37%) to 7.4 ± 0.1 at 21°C. The medium was then sterilised by autoclaving for 20 min at 121°C and a pressure of 15 psi. Glucose was not autoclaved owing to its heat sensitivity, but it was added to the previously autoclaved solution using sterilised syringe filters with a pore size of 0.02 μm.

_E. coli_ (ATCC 25922), obtained from the American Type Culture Collection, was used for this study. For each experiment, bacterial cells were taken from an uniform stock stored in 15% glycerol at –80°C and were transferred to 100 ml of sterile M9 in a 250 ml glass bottle. Cultures were incubated overnight at 37°C on a rotary shaker at 110 rpm. The overnight culture was subcultured into fresh M9 medium and incubated at 21°C until the exponential phase was reached as determined from growth curves and corresponding to an optical density of 0.3 measured at 600 nm using a spectrophotometer (CARY 50 BIO, Agilent, Forest Hill, Victoria, Australia). The number of viable bacterial cells was calculated via the spread plate method using nutrient agar (CM0003, Oxoid, Basingstoke, UK) at the start of each immersion test and after each seven-day period prior to medium replenishment during the longer term immersion tests.

### Table 1. Elemental composition in weight per cent of the different grades of carbon steel used in this work.

| Steel grade | Fe  | C   | Mn  | Si  | S   | P   | Al |
|-------------|-----|-----|-----|-----|-----|-----|----|
| 1010        | Bal.| 0.10| 0.44| <0.02| 0.01| 0.02| 0.035|
| 1020        | Bal.| 0.17| 0.64| 0.18| 0.02| 0.02| <0.005|
| 1030        | Bal.| 0.30| 0.70| 0.25| 0.03| 0.01| <0.005|
| 1045        | Bal.| 0.51| 0.83| 0.15| 0.02| 0.01| <0.005|
To investigate the influence of electrochemical polarisation on bacterial attachment a test was performed with the samples set to a fixed potential. A potentiostat (PARSTAT 2273, AMETEK, Berwyn, PA, USA) was used with the different grades of CS as the working electrode and an Ag/AgCl reference electrode saturated with KCl. These studies were conducted using duplicate steel coupons of each steel grade. For each steel coupon, an individual sterile polypropylene container was used. The volume of test medium (M9) inoculated with *E. coli* was 45 ml. The extent of bacterial attachment on the different grades of CS was measured at a potentiostatically applied potential of –0.71 V for 30 min. This potential was found to be equivalent to the OCP of carbon steels in artificial seawater (data not shown). Potentiostatic polarisation was started directly following the immersion of working electrodes into the M9 medium inoculated with *E. coli*.

**Bacterial attachment and microbial corrosion tests**

Prior to testing, coupons were ultrasonically cleaned with acetone, followed by sterilisation via immersion in absolute ethanol and then aseptically dried within a level 1 physical containment (PC1) cabinet. For initial bacterial attachment tests, the coupons were aseptically introduced into individual sterile polypropylene containers (LS26-60L, ProSciTech, Townsville, Queensland, Australia) containing 45 ml of the M9 medium inoculated with *E. coli*. Control experiments were performed by immersing metallic coupons in abiotic test medium. Triplicate steel coupons were tested for each exposure time, after which the tests were repeated to give *n* = 6. During attachment tests, the coupons were fully immersed in the medium with the polished side facing upward. Coupons were removed from the medium after 15, 30 and 60 min to observe the early stages of bacterial attachment. All tests were carried out in static batch mode at 21°C.

![Figure 1. Light microscope images of etched carbon steel coupons showing differences in grain size, pearlite phase (black grains) and ferrite phase (white grains) between (a) 1010, (b) 1020, (c) 1030 and (d) 1045 steel.](image-url)
To image the initial bacterial attachment, the coupons were removed from the test medium and gently rinsed with sterile saline to remove loosely attached bacterial cells, followed by two rinses with sterile deionised (DI) water (Yuan et al. 2008; Javed, Stoddart, McArthur et al. 2013). Samples were then air dried in a PC1 cabinet at room temperature. Initial bacterial attachment (≤ 60 min) on the surface of the polished coupons was examined using a 3-D optical surface profiler (Contour GT-K1, Bruker) and an optical microscope (MEF4 M, Leica, Vienna, Austria) as described elsewhere (Javed, McArthur, Stoddart et al. 2013; Javed, Stoddart, McArthur et al. 2013). Images were recorded at 20 different locations on each coupon using the 3-D profiler. Bacterial attachment tests carried out at a fixed potential for 30 min indicated a small amount of extracellular polymeric substances (EPS) on the coupon surfaces, which prohibited the counting of individual bacterial cells using the 3-D optical profilometer technique. Therefore, a scanning electron microscope (SEM) technique, as described in more detail later, was used to image the bacterial cells attached to the steel surfaces. Previous work has shown that the SEM and 3-D optical profiler techniques provide comparable counts of surface-attached bacterial cells (Javed, McArthur, Stoddart et al. 2013). Images were taken at 10 different locations on each coupon with the SEM and bacterial cells were counted manually using Image J software (National Institutes of Health) (Javed, McArthur, Stoddart et al. 2013).

For longer term immersion studies (28 days), the polished metal coupons were aseptically introduced into individual sterile 500 ml glass bottles (Schott Durant, ThermoFisher, Scoresby, Victoria, Australia) containing 400 ml of M9 test medium inoculated with E. coli. The coupons were hung vertically on Nylon string using the holes drilled at the top of the coupons. The coupons were fully immersed in the medium throughout the testing. To maintain the bacterial cells in the steady-state growth phase throughout the study period, a semi-continuous mode of E. coli culture was employed, ie 75% of the medium was replaced with an equal amount of a fresh sterile medium every seven days. The coupons remained fully immersed in the medium during the process of replacing the culture medium. Control experiments were performed by immersing a second set of coupons in abiotic M9 medium. The bulk pH of the test medium was measured at the start and end of each immersion test (PHM210, MeterLab, Murfreesboro, TN, USA). These studies were carried out in static batch mode at 21°C. The coupons were retrieved from the media after immersion for 28 days.

The biofilm formation and corrosion features of the coupons from the longer term tests were examined by SEM (SUPRA 40VP-25-38, Zeiss, Oberkochen, Germany) at 3 kV, with magnifications between 500 × and 15,000 ×. To study biofilm formation using the SEM, biotic coupons were gently rinsed as described earlier and then fixed with 2.5 vol.% solution of glutaraldehyde in phosphate buffered saline for 30 min at 21°C. Thereafter, the coupons were removed from the glutaraldehyde solution and washed twice with DI water, followed by stepwise dehydration with 25, 50, 75, 90, and 100 vol.% ethanol for 10 min each. The coupons were then dried in a PC1 cabinet prior to SEM imaging.

Following the measurements of initial bacterial attachment locations and biofilm formation, an ultrasonic treatment was used to remove the attached bacterial cells and/or biofilm formed on the coupon surfaces. This was achieved by placing the coupons in a beaker containing 100 ml of Clarke’s solution, which is a mixture of concentrated HCl, 2% antimony trioxide and 5% stannous chloride, and ultrasonically cleaning for 1 min (Unisonics Model FXP-10D, Brookvale, New South Wales, Australia). Clarke’s solution is passive to carbon steel and does not affect the coupons during the cleaning process (ASTM 2011c). The coupons were then rinsed with DI water followed by an ethanol rinse and dried using a nitrogen gas flow.

The cleaned metallic coupons were analysed using an optical microscope, 3-D optical profilometer and SEM to observe the effect of bacterial attachment and corrosion on the surface of the coupons. For the initial bacterial attachment samples an additional step was undertaken to determine the attachment location relative to the microstructure. These coupons were subsequently etched using 2% Nital solution for 15–20 s. After etching, the microstructures of the metallic coupons were again recorded using the 3-D optical surface profilometer and optical microscope at the same locations used for the initial bacterial attachment imaging. Finally, the images of bacterial attachment were processed using image processing software and overlapped at the same location on the metal microstructure, therefore demonstrating the correlation between the microstructure and attachment locations as described previously (Javed, McArthur, Stoddart et al. 2013).

**Corrosion rate calculations**

The extent of corrosion was determined by measuring the weight loss using a high accuracy mass balance (Mettler Toledo MS205DU, readability 0.01 mg) and then calculating the corrosion rate by the gravimetric method as per ASTM standard G1–03 (ASTM 2011c):

\[
\text{Corrosion rate}(\mu m \, y^{-1}) = \frac{8.76 \times 10^7 \times \text{weight loss(g)}}{\text{density(g cm}^{-3}) \times \text{area(cm}^2) \times \text{time(h)}}
\]
The analysis of bacterial cell attachment on the different grades of CS was performed for 15, 30 and 60 min exposures. The number of bacterial cells attached to CS coupons of all grades increased with immersion time (Figure 2a). However, the rate and magnitude of bacterial attachment depended on the grade of the CS. A distinct difference was observed in the number of bacterial cells attached to the different steel types over time. The bacterial cells attached to 1010/1020 steel grades increased in an approximately linear fashion with time, whereas the number of cells attached to 1030/1045 steel grades showed only a very small increase between 30 and 60 min immersion times. Statistical analysis showed a significant difference ($p < 0.05$) in the average number of bacterial cells attached to the different CS coupons after immersion for 60 min. The number of bacterial cells attached after 60 min decreased with the increasing pearlite phase content of the CS (Figure 2b).

The spatial distribution of the initial bacterial attachment on different grades of CS coupons after 30 min is shown in Figure 3, indicating a relatively uniform distribution of bacterial cells.

**Electrochemical polarisation and bacterial attachment**

Studies of the attachment of bacterial cells on the four different grades of CS coupons at the same applied potential...
Figure 2. (a) Number of bacterial cells retained on the four different grades of CS coupons as a function of time in M9 medium inoculated with *E. coli*. (b) Relationship between the pearlite phase content of each steel type and the total number of bacterial cells attached to the different grades of CS after immersion for 60 min.
Notes: Interpolating splines and an exponential fit have been added as a guide to the eye only. Data shown: mean ± SE, *n* = 120.

Figure 3. 3-D optical profilometer images after immersion for 30 min showing initial attachment of bacterial cells (red) to the different grades of CS, ie (a) 1010, (b) 1020, (c) 1030 and (d) 1045 in M9 medium inoculated with *E. coli*. 
Steel, despite the grain size of the 1020 grade steel being 20% larger. In general however, the percentage of total bacterial cells attached on grain boundaries was found to decrease linearly with increasing grain size of the CS coupons (Supplementary material; Figure S1).

Longer term immersion tests (28 days)

Longer term immersion tests showed significantly increased corrosion in all grades of CS immersed in the M9 medium in biotic conditions, compared to abiotic conditions (Figure 7). Statistical analysis showed no significant difference ($p > 0.05$) in the corrosion rate of the different grades of CS in abiotic conditions. Under biotic conditions, there was no significant difference ($p > 0.05$) observed in the corrosion rates between the 1010 and 1020 steel coupons or between the 1020 and 1030 steel coupons. However, a significant difference ($p < 0.05$) was found in the corrosion rates between the 1010 and 1030 steel coupons and between the 1045 and all other grades of CS coupons tested. Overall the corrosion rate of the different grades of CS coupons immersed in the biotic M9 medium was found to increase linearly with the pearlite phase content of the steel.

SEM images of the biofilm and corrosion product formed on the different grades of CS coupons after immersion for 28 days in M9 medium under biotic and abiotic conditions are shown in Figure 8. Figure 8a–d shows the formation of corrosion products on the surface of the CS coupons in the abiotic test medium. Figure 8e–h shows the bacterial attachment and EPS production on the surface of the CS coupons in the biotic test medium. To reveal the extent of corrosion damage, the coupons were cleaned with Clarke’s solution as described earlier and analysed via SEM. The results showed less attack on the surface of the CS coupons immersed in the abiotic test medium (Figure 8i–l) compared to the coupons immersed in the biotic test medium (Figure 8m–p).

Correlation between initial bacterial attachment and substratum microstructure

The correlation between initial bacterial attachment and the metal microstructure was examined after immersion for 30 min with no applied potential by superimposing the 3-D optical profilometry images of bacterial attachment and microscopic images of the underlying metal microstructure, as shown in Figure 6. The results showed a preferential attachment of bacterial cells to the grain boundaries. No obvious correlation was found between the total number of attached bacterial cells and the average grain size of the different grades of CS coupons. For example, the 1020 grade steel had $5 \times$ more bacteria attached after immersion for 60 min compared to the 1030 grade steel, despite the grain size of the 1020 grade steel being 20% larger. In general however, the percentage of total bacterial cells attached on grain boundaries was found to decrease linearly with increasing grain size of the CS coupons (Supplementary material; Figure S1).

pH and viable bacterial counts

The bulk pH and number of viable bacterial cells (CFU ml$^{-1}$) in the test medium was measured after every seven days, prior to medium replenishment, to determine whether there was any relationship between these parameters and corrosion (Table 4). Under abiotic conditions, no change in the pH of the medium was observed over time (data not shown). In the case of biotic conditions however, the pH had decreased significantly from the initial value of 7.4 when measured after immersion for the first seven days and was observed to be same for the subsequent measurements up to 28 days. The CFU ml$^{-1}$ was determined in order
Biofouling affected the initial bacterial attachment as well as the subsequent longer term corrosion. The surface roughness of the substratum has been reported to affect the initial bacterial attachment and subsequent biofilm formation (Hilbert et al. 2003; Crawford et al. 2012). In order to minimise the influence of surface roughness in the current study, coupons were polished to a 0.02 μm surface finish. The surface roughness analysis results showed that there was no significant difference between the polished surfaces of the different grades of carbon steel coupons for large scanned areas. For the smaller scanned areas a relatively small but statistically significant difference was found in the average surface roughness values of the 1010 and 1045 steel coupons. This slight difference might be due to the difference in the hardness of two steel grades, corresponding to the to evaluate the microbial population with time and to provide insight into possible population size effects on the corrosion behaviour of the steel coupons. The results showed no significant change in the number of viable bacterial cells in the test medium over the course of the long term immersion studies.

Discussion

In this study, the effects of composition, microstructure and applied potential on the initial attachment of E. coli cells (≤ 60 min) and the subsequent longer term (28 days) corrosion of CS were investigated. Four different grades of CS coupons were mirror polished and immersed in M9 medium with and without E. coli for different time intervals. The results indicated that the grade of CS significantly affected the initial bacterial attachment as well as the subsequent longer term corrosion.

The surface roughness of the substratum has been reported to affect the initial bacterial attachment and subsequent biofilm formation (Hilbert et al. 2003; Crawford et al. 2012). In order to minimise the influence of surface roughness in the current study, coupons were polished to a 0.02 μm surface finish. The surface roughness analysis results showed that there was no significant difference between the polished surfaces of the different grades of carbon steel coupons for large scanned areas. For the smaller scanned areas a relatively small but statistically significant difference was found in the average surface roughness values of the 1010 and 1045 steel coupons. This slight difference might be due to the difference in the hardness of two steel grades, corresponding to the

Figure 5. SEM images after 30 min of immersion showing initial attachment of bacterial cells (black) to different grades of CS, ie (a) 1010, (b) 1020, (c) 1030 and (d) 1045 at the same applied potential (–0.71 V) in M9 medium inoculated with E. coli.
The bacterial attachment studies showed that the number of bacterial cells attached to the CS coupons increased with immersion times up to 60 min. However, in the absence of an applied potential, the total number of bacteria attached was significantly different on the different grades of CS. These results are consistent with previous studies which showed that the attachment of bacteria was influenced by the chemical composition of the substrate (Beech & Gaylarde 1989). The main difference in the chemical composition of the four different grades of CS coupons tested in the present work was the carbon content. The percentage of carbon directly relates to the formation of the pearlite phase in that the higher the carbon percentage, the greater the amount of the pearlite phase present. The bacterial attachment results showed a drop in the number of attached cells with increasing different levels of carbon content (Krauss 2003). However, the initial bacterial attachment results showed no overall correlation with the surface roughness parameters of the different steel grades. This suggests that other factors, such as metal microstructure, played a more significant role in the initial bacterial attachment to these surfaces compared to the relatively small differences observed in surface roughness.

The bacterial attachment studies showed that the number of bacterial cells attached to the CS coupons increased with immersion times up to 60 min. However, in the absence of an applied potential, the total number of bacteria attached was significantly different on the different grades of CS. These results are consistent with previous studies which showed that the attachment of bacteria was influenced by the chemical composition of the substrate (Beech & Gaylarde 1989). The main difference in the chemical composition of the four different grades of CS coupons tested in the present work was the carbon content. The percentage of carbon directly relates to the formation of the pearlite phase in that the higher the carbon percentage, the greater the amount of the pearlite phase present. The bacterial attachment results showed a drop in the number of attached cells with increasing different levels of carbon content (Krauss 2003). However, the initial bacterial attachment results showed no overall correlation with the surface roughness parameters of the different steel grades. This suggests that other factors, such as metal microstructure, played a more significant role in the initial bacterial attachment to these surfaces compared to the relatively small differences observed in surface roughness.

Figure 6. Superimposed 3-D profilometer images of attached bacterial cells (red) and the underlying metal microstructure of (a) 1010, (b) 1020, (c) 1030 and (d) 1045 CS coupons after immersion for 30 min in M9 medium with *E. coli* cells.

Figure 7. Relationship between the pearlite phase content and the corrosion rate of different grades of CS tested in M9 medium after immersion for 28 days. Data shown: mean ± SE; n = 6.
local electrochemical (micro-galvanic) potentials on the surface of the steel (Gutman et al. 1972), which might in turn have influenced the attachment of bacterial cells to these surfaces.

In order to eliminate the intrinsic micro-galvanic potential variations on the surface of different CS grades, the pearlite phase content of the CS. The exact reason for the reduced levels of attachment is currently unknown, but it could be related to micro-galvanic cells formed between the pearlite and ferrite phases. The pearlite phase acts as a cathode to the anodic ferrite phase. The increase in the percentage of pearlite relative to ferrite would change the local electrochemical (micro-galvanic) potentials on the surface of the steel (Gutman et al. 1972), which might in turn have influenced the attachment of bacterial cells to these surfaces.

In order to eliminate the intrinsic micro-galvanic potential variations on the surface of different CS grades,
initial bacterial attachment studies were also undertaken at a fixed externally applied potential. This test showed that there was considerably less variation in the number of attached bacterial cells when the different grades of steel were held at a constant potential, in comparison to the case without an applied potential. These findings are consistent with previous studies which showed that an externally applied potential on a metal surface can affect the attachment of bacterial cells and subsequent biofilm formation, depending on the extent and polarity of the potential (Busalmen & Sánchez 2005; Schaule et al. 2008; Duan & Lin 2011; Nithila et al. 2012).

A number of mechanisms have been suggested for the observed changes in initial bacterial attachment due to externally applied potentials on metallic substrata. These mechanisms include a localised pH change at the metal/solution interface, the bactericidal action of metal ions, electrostatic interactions, and/or modification of conditioning film formation (Armon et al. 2001; Busalmen & Sánchez 2005; Duan & Lin 2011). While it was beyond the scope of the current investigation to find out the exact mechanism(s) involved in the observed differences in initial bacterial attachment on the different steel grades with/without an externally applied potential, this would be an interesting topic for future studies. Furthermore, it would be interesting to identify the local micro-galvanic potentials on the different steel grades and to explore their correlation with bacterial cell attachment.

The average grain size of the four different grades of steel coupon was measured and compared with the total number of bacterial cells initially attached (without applied potential), with no consistent trend observed. The number of total bacterial cells attached showed a pattern of 1010 > 1020 > 1030 > 1045, while the grain size showed a trend of 1045 > 1020 > 1030 > 1010. These results, especially for the 1020 and 1030 grade steels, are somewhat in contrast to previous studies which showed greater bacterial cell attachment on smaller grain size materials compared to larger grain size materials (Sreekumari et al. 2001; Javed, Stoddart, McArthur et al. 2013). However, in those previous studies, the grain size difference was for the same substratum chemical composition. In the present study, both the grain size and the chemical composition of the substratum were changing. This suggests that the chemical composition and the pearlite phase content of the CS coupons have a greater effect on the initial bacterial attachment than the average grain size.

The comparison between the location of initial bacterial attachment (without applied potential) and the underlying metal microstructure showed that bacterial cells attached preferentially on the grain boundaries, matching previous studies (Geesey et al. 1996; Kielemoes et al. 2000; Javed, Stoddart, McArthur et al. 2013). The preferential attachment of bacterial cells at grain boundaries could be related to factors such as the difference in elemental composition, surface energy variations and/or anodic dissolution of grain boundary sites, as discussed in detail elsewhere (Javed, Stoddart, McArthur et al. 2013).

Longer term (28 days) immersion studies showed no significant difference in the corrosion rates of the four different grades of CS in the M9 medium under abiotic conditions. Compared to the abiotic conditions, a significant increase was observed in the corrosion rates of the four grades of steel tested in M9 medium under biotic conditions. The increased corrosion rate could either be due to the consumption of the phosphate (a known corrosion inhibitor) in the test medium by the bacterial cells and/or production of acidic by-products by the bacteria in the presence of glucose (Rodin et al. 2000). The bulk pH of the medium was monitored at the end of each immersion test. The results showed a significant drop in the pH value (Table 4) over time, confirming that the bacterial cells produced acidic by-products in the M9 medium.

The corrosion rates of the different grades of CS in this study were found to increase with the pearlite content under biotic conditions. Interestingly, these results are consistent with a previous study which used a completely different bacterial strain, i.e. the sulphate-reducing bacterium (SRB) Desulfovibrio vulgaris, and showed that the corrosion rate increased linearly with the percentage carbon content of the steel coupons (Mara & Williams 1972). In contrast, Ashton et al. (1973) found no correlation between the carbon content and corrosion when studying six different iron-carbon alloys in the presence of the bacterium E. coli in anaerobic conditions. At present the cause of these different correlations between carbon content and corrosion rate is unclear. As was found in the current work, a number of mechanisms are often in play in MIC tests and it is possible that another factor was masking the dependence on carbon content in the study by Ashton et al. (1973).

There are conflicting reports on whether the number of initially attached bacterial cells relates to subsequent levels of MIC attack on metal surfaces. Gaylarde and Johnson (1980) showed that preventing the attachment of SRB to a metal surface decreased the rate of corrosion of that metal. Likewise, Al-Shamari et al. (2013) observed higher levels of corrosion for greater population densities of aerobic, anaerobic and SRB in different oilfield water handling systems. However, in the present study, the number of bacterial cells initially attached showed no correlation with the longer term (28 days) corrosion of the different grades of CS. These findings are consistent with previous studies which showed no correlation between either the number of bacterial cells initially attached to steel surfaces or the general microbial population in the test medium and the observed corrosion of CS coupons (Beech et al. 1994; Zintel et al. 2003; Javed et al. 2014).
Conclusions

The results reported in this paper indicate that the chemical composition and microstructure of different grades of CS can influence initial bacterial attachment and subsequent corrosion in the presence of E. coli. The number of attached bacterial cells was different for different grades of CS and decreased with increasing pearlite phase content of the CS. The variation in the number of bacterial cells attached was significantly reduced on different grades of CS coupons held at a fixed applied potential compared to the steel samples without any applied potential. There was no consistent relationship between the number of bacterial cells initially attached and the average grain size of the CS grades tested. The longer-term immersion studies showed increased corrosion rates for all grades of CS coupons in the biotic M9 medium compared to the abiotic M9 medium. The corrosion rate of the CS grades increased linearly with the pearlite phase content. Overall the results showed that the carbon content of the steel can have a significant effect on initial bacterial attachment and the subsequent longer term corrosion.

The present results showed the importance of material composition and/or microstructure on the initial attachment of bacteria. Further work is needed to help clarify the exact reason(s) for the differences in bacterial cell attachment observed on the four different grades of CS. Studies aimed at answering these questions are currently in progress and it is hoped that they will provide additional information to support the development of mitigation strategies for bacterial attachment and the resulting corrosion, for example, by providing suggestions for appropriate alloying additions and/or processing techniques for the materials.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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