The influence of the surface condition on the adhesion of *Pseudomonas fluorescens* (ATCC 17552) to copper and aluminium brass

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Abstract

The adhesion of *P. fluorescens* (ATCC 17552) to copper, zinc and aluminium brass in the absence and presence of a surface oxide layer was evaluated performing comparative batch experiments with various times of exposition. The adhesion of this strain to bare copper and aluminium brass was extremely low even after 120 min of exposition. On aged samples, on the other hand, adhesion increased with time on both metals. The degree of bacterial adhesion to aged metals was found to follow the sequence zinc > aluminium brass > copper, and the differences were related to the composition of the oxide layer. The presence of ZnO in the layer strongly increased the adhesion due to the large co-ordination capacity of Zn^{2+} ions. Correlation was found between the water contact angle of the metal surface and the degree of bacterial adhesion showing that adhesion increases with the decrease in the contact angle. Bacteria were found to have a hydrophilic surface with a water contact angle of $23.5 \pm 2^{\circ}$ as determined by the sessile drop method. © 2002 Published by Elsevier Science Ltd.

Keywords: Bacterial adhesion; Metal oxides; Copper alloys

1. Introduction

Adhesion of bacteria to solid surfaces is a general phenomena which is recognised as the first step in the development of biofilms. In particular, the growth of biofilms on metal surfaces leads to changes in the rate of corrosion process, usually inducing a localised attack and reducing the service life of industrial devices such as tanks, conducting pipes and heat exchangers, among others (Sánchez and Schiffrin, 1985; Sand, 1997).

Bacterial adhesion depends on the physicochemical properties of both, the substratum material and the bacterial cell surface. Surface hydrophobicity and electrostatic charge have been proposed as the main properties determining bacterial adhesion and its participation in the adhesion process has been interpreted using the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloidal stability (Norde and Lyklema, 1989). This theory summarises the van der Waals and the electrostatic contributions to the interfacial interaction energy between two interacting surfaces, and has been demonstrated to be useful to explain most of the bacterial adhesion results (Marshall et al., 1971; Busscher and Weerkamp, 1987; van Loosdrecht et al., 1989).

Deviations from the DLVO behaviour have been reported in some cases being mainly related to the occurrence of specific interactions at a very short separation distance (Jucker et al., 1998; Busalmen and Sánchez, 2001).

The possibility to form specific bonds between bacteria and metals depends on both the metal surface chemical composition and the composition of the outermost layer of bacterial cells. Metals are generally covered by a layer composed of oxides and hydroxides which are the product of metal ions interaction with the surrounding electrolyte. The formation of this layer modifies the physicochemical properties of the surface, including charge and hydrophobicity. Gram (−) bacteria on the other hand are generally covered by an extracellular polysaccharide layer which often carries phosphate and carboxylic acid groups (White, 1995).

The aim of this work was to determine the influence of surface oxides on the adhesion of bacteria to copper and aluminium brass. Aluminium brass is widely used in the construction of heat exchanger tubes, which are often affected by microbiologically influenced corrosion (Sánchez and Schiffrin, 1985). Copper, on the other hand, is used in the construction of potable water distribution systems, which are frequently infected by bacteria (Bremer and Geesey, 1991; Siedlarek et al., 1994; Sequeira, 1995). The influence of zinc as the main alloying element was investigated.

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Polyethylene was taken as a reference material since it has been widely used as a probe in bacterial adhesion research (Fletcher and Loeb, 1979; Pringle and Fletcher, 1982; Rogers et al., 1994).

2. Materials and methods

2.1. Biological material

Pure cultures of *P. fluorescens* (ATCC 17552) were grown at $32°C$ with continuous shaking in a minimal broth containing 0.06% peptone in artificial tap water (ATW). The mineral base composition was $MgSO_4$ $(40\ mg\ l^{-1})$, $MgCl_2$ $(60\ mg\ l^{-1})$, KNO_3 $(25\ mg\ l^{-1})$, $CaCl_2$ $(110\ mg\ l^{-1})$, Na_2CO_3 $(560\ mg\ l^{-1})$ and $NaNO_3$ $(20\ mg\ l^{-1})$ in distilled water; the pH was adjusted to 7.6. Cells were harvested from cultures at the mid-exponential phase of growth by centrifugation for 10 min at $10\ 000\ g$ in a Jouan BR4i refrigerated centrifuge, washed, and suspended in ATW after centrifuging again.

2.2. Metal samples preparation

Disc samples of aluminium brass (Al–brass, UNS 68700), copper and zinc were included in fast curing acrylic resin on appropriate PVC holders with an electric contact on the back. Samples were abraded to grade 600 with emery paper, and mirror polished with 0.05 μm alumina powder (Type B-Buehler, Lake Bluff, USA). After that, samples were rinsed gently with distilled water.

Bare and aged samples were used. Bare samples correspond to those used immediately after polishing and rinsing. This condition is assumed to correspond to a metal surface uncovered or patchily covered by an oxide film.

Aged samples were prepared by immersion of polished samples during 24 h in ATW. A continuous oxide layer usually covers the metal surface after this treatment.

Polyethylene (PE) samples were included as reference surfaces. PE samples were immersed consecutively in 1% NaOH at $60°C$ for 10 min, and in 5% HCl at $60°C$ for 2 min. Before use samples were rinsed gently with distilled water.

2.3. Adhesion assay

Samples of each metal and those of PE were exposed to cell suspensions with a cell density of 1×10^7 cells ml^{-1} in stagnant conditions at room temperature. Samples were withdrawn after various exposition times (30, 60, 90 and 120 min) and washed gently by immersing in distilled water to eliminate reversibly adhered bacteria. Irreversibly adhered cells were fixed by drying with hot air, and stained for 1 min with crystal violet. Microscopic cell counts were carried out with an Olympus PMG3 Metallurgical Microscope at $500 \times$ magnification. Ten different fields of $0.036\ mm^2$ were randomly selected on each sample to give statistical significance to the obtained values. Special care was taken to select sampling areas distant from the edge of the sample to eliminate distortion of results. Although, only one physical sample was extracted for each data point during the experiments, the presented data are the result of not < 10 replicates for each condition on each different material, giving confidence to the obtained information. Typical results are presented showing the tendency obtained throughout the experiments. Data were expressed as the per centage of adhesion taking the maximum bacterial counts as 100%.

2.4. Hydrophobicity measurements

The bacterial cell surface hydrophobicity was determined by both the measurement of the water contact angle (θ_w) of bacterial layers following the technique described by Busscher and Weerkamp (1987), and the determination of the microbial adhesion to hydrocarbons (MATH) using *p*-xylene as the extracting solvent (Rosenberg et al., 1980).

Contact angles were calculated using the sessile drop method as (Gotowski, 1991)

$$\theta_w = 2\tan^{-1}(h/r),$$

where h and r are the height of the drop and the radius of the drop contact circle, respectively. For the determination of bacterial adhesion to hydrocarbons, 3 ml of a phosphate buffered saline (PBS) bacterial suspension were vigorously vortexed in the presence of increasing volumes of *p*-xylene during 120 s. The emulsion formed was left at rest for 15 min to allow the complete separation of the two phases. The absorbance at 600 nm was measured on both the initial cell suspension (A_0) and the lower aqueous phase after the emulsion separation (A). Results are presented as percentage of cells retention in the organic phase $(\%B)$ as a function of the volume of *p*-xylene added as (Kiremitçi-Gümüsderelioglu and Pesmen, 1996)

$$\%B = [1 - (A/A_0)] \times 100.$$

The surface hydrophobicity of bare and aged metal samples was also determined by measuring the water contact angle using the sessile drop method. Before measurements, samples were rinsed with ethanol to remove residual water, and then dried with cool air during 30 s. Contact angles were measured by triplicate at room temperature $(25\pm2°C)$ (Feng et al., 1998).

2.5. UV–visible reflectance spectroscopy

UV–visible absorption spectra of surface oxides grown on aluminium brass, copper and zinc after the ageing treatment were recorded in situ in a Shimadzu UV-160A double beam spectrophotometer with a differential reflectance device (Sánchez et al., 1991). Baseline correction was made polarising two identical polished surfaces to a negative cathodic potential to prevent oxides growth (Al–brass and Cu electrodes were polarised to -0.8 V using a saturated
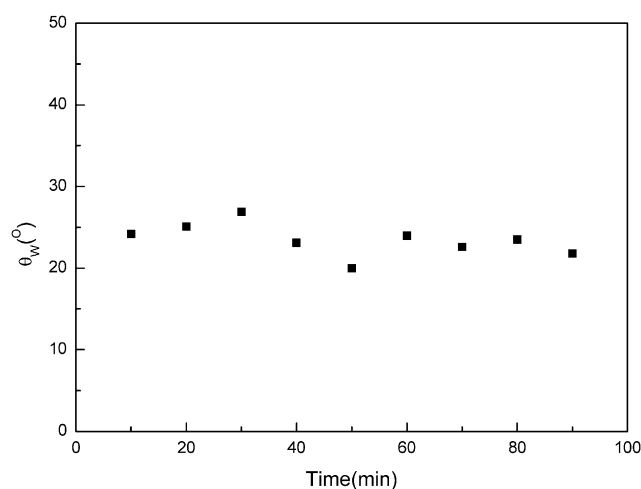
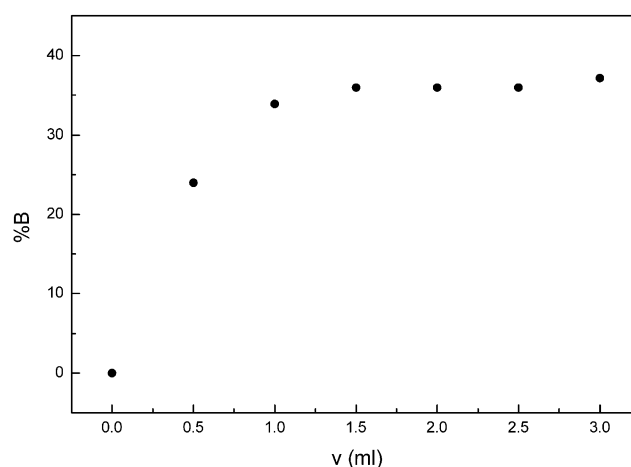Fig. 1. Time dependence of water contact angle on *P. fluorescens* layers.

Fig. 2. Retention of bacteria in the organic phase (%*B*) as a function of the volume of *p*-xylene added during MATH experiments.

Calomel electrode (SCE) as a reference. Zn electrodes were polarised to −1.5 V SCE).

The electrode in the sample compartment was left at its corrosion potential during 24 h to allow oxides growth (ageing treatment). The oxides absorption spectra between 200 and 800 nm were recorded at the end of the ageing stage period.

3. Results

3.1. Bacterial surface hydrophobicity

Determination of the water contact angle of bacterial surface by the sessile drop method is affected by the drying time of the bacterial layer (van Oss, 1978; Absolom et al., 1983; Busscher et al., 1984). In order to determine the appropriate time to measure the angle value, the dependence of the contact angle of layers of the strain used with the drying time was determined. Results in Fig. 1 showed that over a 100 min period there was no significant variation in the contact angle value. The mean value was determined to be $23.5 \pm 2°$, in agreement with the previously reported results (van Loosdrecht et al., 1987). Bacteria showing a θ_w lower than 30° are usually considered as hydrophilic ones (van der Mei et al., 1998). The hydrophilic character of the strain was also evidenced by MATH experiments (Rosenberg et al., 1980), where a maximum retention of the bacterial population in the organic phase (%*B*) of about 35% was obtained (Fig. 2).

3.2. Metal surface characterisation

The water contact angle of metal surfaces in the bare and the aged condition was determined (Table 1). This angle is an indirect measurement of the surface hydrophobicity and has been successfully used to predict adhesion of bacteria

Table 1
Water contact angle (θ_w) of the different surfaces under study. Results are indicated as mean value $\pm$ standard deviation ($n = 3$)

Sample	θ_w (°)
Aged Zn	0.0 ± 0
Aged Al–brass	29.7 ± 0.2
Aged Cu	42.5 ± 0.6
Bare Cu	45.9 ± 1.3
Bare Al–brass	51.2 ± 1
PE	96.7 ± 0.5

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to polymeric materials (Fletcher and Loeb, 1979; Pringle and Fletcher, 1982). As can be observed in Table 1 higher contact angles for metals surfaces were those corresponding to bare aluminium brass and copper, with values of 51.2° and 45.9°, respectively. The ageing treatment results in a strong decrease of the contact angle of aluminium brass up to a value of 29.7°. Meanwhile, in the case of copper, the obtained value was just slightly lower (42.5°) than the one measured on the bare surface.

On zinc samples the instantaneous oxidation of the surface after polishing hindered the determination of θ_w on a bare surface. On aged zinc, on the other hand, the oxide covering the surface was completely hydrophilic and rendered a value of $\theta_w = 0°$.

The water contact angle for PE was also determined and included for comparison (Table 1).

Despite the relative differences of θ_w values measured on the various metals and conditions, it is important to note that with the only exception of PE, contact angles were always below 65°, which is recognised as an indication of the hydrophilic character of the surface of materials (Vogler, 1998) (see below).
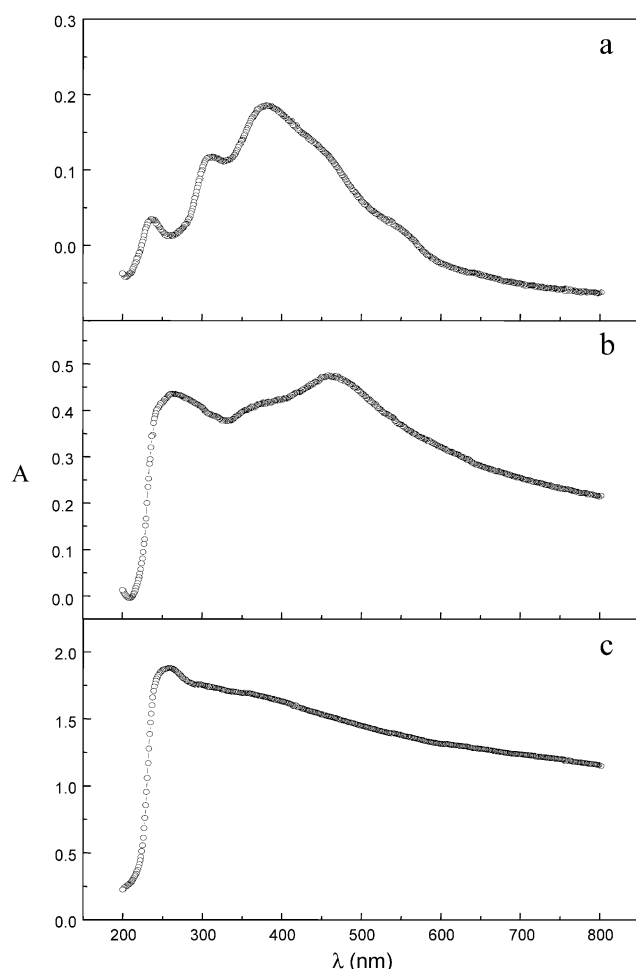
Fig. 3. Differential absorbance spectra of: (a) Cu; (b) Al–brass; and (c) Zn, after 24 h of immersion in ATW at the corrosion potential.
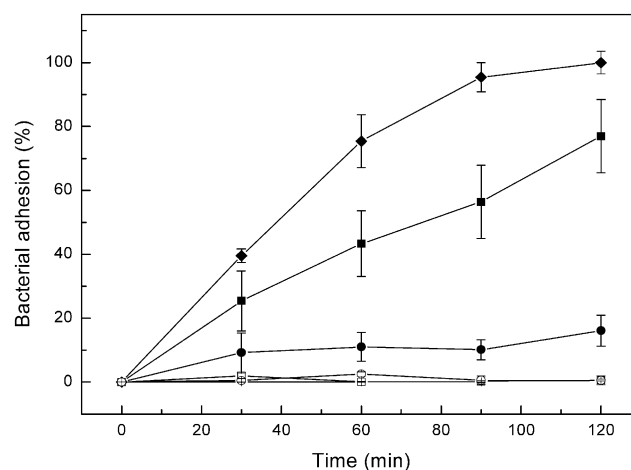

Fig. 4. Bacterial adhesion (%) to various materials as a function of time: (♦) aged Zn; (▨) aged aluminium brass; (●) aged Cu; (○) bare Cu; (□) bare aluminium brass; and (+) PE. 100% of adhesion = 325 adhered bacteria/100 μm^2. Results are indicated as mean value ± standard deviation ($n = 10$).

1   In addition to θ_w measurements, oxides grown during the ageing treatment were also characterised by differ-
3 ential reflectance spectroscopy. Results are presented in Fig. 3. On copper, the presence of absorption peaks at 237,
5 314 and 380 nm, and shoulders at 462 and 560 nm indicates the participation of Cu_2O in the passivating film as
7 a main component (Fig. 3a) (Hummel, 1983; Sánchez et al., 1991). On aluminium brass (Fig. 3b), the occurrence of
9 peaks at the same wavelengths but with different intensities was observed. A broad peak at 462 nm, a minor peak at
11 360–380 nm and a shoulder at 237 nm suggest the presence of Cu_2O. A peak at 260 nm was also detected. This peak
13 was also found on aged zinc (Fig. 3c), and indicates the formation of zinc oxo-hydroxides (Kim et al., 1995).

15 *3.3. Bacterial adhesion*

   Metal samples were exposed during different time periods
17 to bacterial suspensions in order to determine the adhesion of cells to the various kind of surfaces. As can be observed in
19 Fig. 4, there was almost no bacterial adhesion on bare metal

samples, on which the number of adhered cells was negli-
gible even after 120 min of exposition. Bare zinc was not   21
included in these tests since, as discussed above, it was
not possible to obtain a bare surface in contact with the   23
electrolyte due to its high susceptibility to oxidation.
   The number of bacteria adhering to aged metals, on the   25
other hand, was increasing at different rates with the time
of exposition. Results were expressed as the per centage of   27
adhesion taking the number of bacteria adhered to zinc at
120 min (325 adhered bacteria/100 μm^2) as 100%. Adhe-   29
sion was found to increase faster following the sequence
zinc > Al–brass > copper (Fig. 4). Final adhesion values of   31
16.3–77.5% were obtained for copper and aluminium brass,
respectively.   33
   On the PE reference surface the adhesion was negligible
throughout the experiments (Fig. 4).   35

4. Discussion

   Adhesion of bacteria to the surface of different materials   37
has been extensively studied during the last 25 years. The
emerging conclusion from these studies, mainly those re-   39
lated to polymeric surfaces, was the correlation originally
found by Absolom et al. (1983) between the interfacial sur-   41
face tension of both the material (γ_{sv}) and the bacterial
surface (γ_{bv}), as related to the interfacial surface tension 43
of the surrounding medium (γ_{lv}). For hydrophilic bacteria
($\gamma_{lv} < \gamma_{bv}$) the adhesion decreases as the material surface   45
hydrophobicity increases. In the case of hydrophobic bacte-
ria ($\gamma_{lv} > \gamma_{bv}$), on the other hand, the adhesion increase with 47
the increment of the surface hydrophobicity. Both, the mea-
surement of the water contact angle (Fig. 1), and the MATH   49
tests (Fig. 2) showed that bacterial cells used in this work
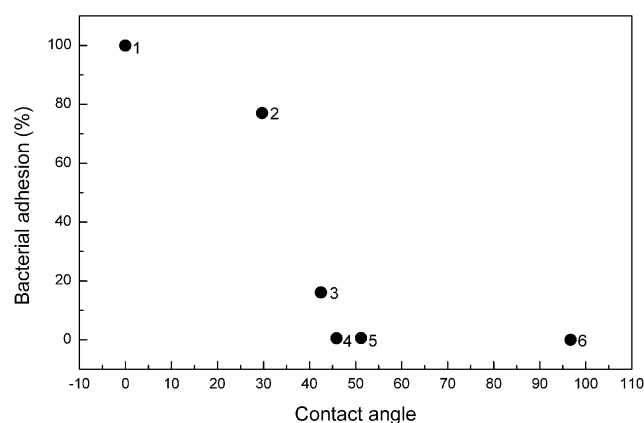are hydrophilic. As shown in Fig. 5, the adhesion of this   51

Fig. 5. Dependence of bacterial adhesion (%) on the water contact angle (θ_w) of the surface: (1) aged Zn; (2) aged aluminium brass; (3) aged Cu; (4) bare Cu; (5) bare aluminium brass; and (6) PE.

strain to various metal surfaces was observed to decrease with the diminution of surface hydrophilicity in the order: aged Zn > aged Al–Brass > aged Cu > Al–Brass > Cu in agreement with the previously cited statement.

In connection with the hydrophobic behaviour of surfaces, another important relation was established regarding the self-association of water at material surfaces (Vogler, 1998). It has been determined that the interaction of water with Lewis acid/base sites on the surface competes with the self-association of water molecules, and follows a linear relation to surface wettability (Vogler, 1998). At a high enough surface density of Lewis sites, the self-association of water can be disrupted. It occurs at a value of $\theta_w = 65°$ for the surface water contact angle. Hydrophobic surfaces exhibit θ_w values higher than $65°$, whereas hydrophilic ones exhibit θ_w values lower than this limit.

Even when metal and metal oxides are typically hydrophilic (Kinloch, 1987) (Table 1), there are differences between them, particularly in their relation with water, depending on the surface chemistry. Values presented in Table 1 showed that oxidised metals are more hydrophilic than bare metals and this is related to the increment in the surface density of acid/base sites during the oxidation process (Sato, 1998).

Adhesion of bacteria to bare metals was negligible (Fig. 4) and this could also be related to a low number of binding sites (acid/base sites) on the surface. Oxides on the other hand, are hydroxilated surfaces, which are able to bond water and other H-bond forming molecules, like those present in the bacterial surface (White, 1995).

Besides the number of binding sites on the surface, a more detailed explanation for results in Table 1 and Fig. 4 regarding the oxidised surfaces should consider the influence of chemical features of the metal ions involved. As the interaction force between a metallic ion and other ion or molecule depends on the ion co-ordination capacity (Cotton and Wilkinson, 1993), differences observed in both, the water contact angle, and the adhesion of bacteria to the

various oxidised surfaces can also be related to variation in the oxide layer composition.

As can be observed in the reflectance spectrum of aged Cu (Fig. 3a), the oxide film on this surface is mainly composed of Cu_2O (Hummel 1983; Sánchez et al., 1991). Aluminium brass, on the other hand, is a copper alloy with a high content of zinc, and as it is shown by the reflectance spectrum in Fig. 3b, the presence of both Cu_2O and ZnO can be detected in the surface oxide layer. ZnO was the only component on the oxidised surface of metallic Zn (Fig. 3c). Whereas little differences exist between the contact angle values on bare and oxidised copper (Table 1), the presence of ZnO in aluminium brass introduces a strong variation on the contact angle of the metal surface after the ageing treatment (Table 1), which results in a value significantly lower than the one for bare aluminium brass. On oxidised zinc, the measured contact angle was null, showing a completely hydrophilic surface (Table 1).

The adhesion of bacteria to the various surfaces showed changes similar to those observed in contact angle values, and were also related to the presence of ZnO in the oxide layer. The adhesion to bare copper and aluminium brass was very low, and increased on oxidised samples, the increment being higher on aluminium brass. The highest adhesion was observed on zinc where the film was only composed of ZnO (Fig. 4).

These results can be explained considering the reactivity of an oxidised surface in which M^{n+} ions have an unsatisfied co-ordination capacity (Cotton and Wilkinson, 1993). Metallic ions with a high charge–volume relation are acid cations which are able to accept electron "pairs" from, and establish strong co-ordination with groups such as O^{2-}, CO, NH^{2-}, OH^- and COO^-. Several of these groups can be found in the outermost layer of bacteria (Jucker et al., 1997). Among these metallic ions, Zn^{2+} has the biggest acid force and exhibit a strong co-ordination capacity with a high reaction rate. A ligand as water can easily get in and out from the co-ordination sphere of Zn^{2+}, allowing an easy exchange with others ligands as those found on the bacterial outermost layer. Cu^{1+}, on the other hand, is a cation with a very low co-ordination capacity due to its low acidity.

Finally, if the oxidation of bare metals during the exposition time to bacterial suspensions is considered, an effect on bacterial adhesion should be observed. However, bacterial adhesion to bare metals was negligible even after 120 min of immersion. This apparent contradiction can be partially explained by our previous results on the effect of bacterial suspensions on the oxide formation process (Busalmen et al., 1998). Heterogeneous oxygen reduction is the controlling reaction of the corrosion process. It has been demonstrated using ellipsometry that the presence of respiring bacteria strongly inhibited the growth of oxides on aluminium brass at open circuit potential as a consequence of the depletion of dissolved oxygen in the bulk solution by cells respiration, and thus preventing bacterial adhesion (Busalmen et al., 1998).

5. Conclusions

Bacterial adhesion to metal surfaces is strongly influenced by the presence of an oxide layer. Changes in the composition of this layer as a consequence of the presence of alloying elements are key factors determining the adhesion process and lead to a faster and increased colonisation of the surface, when the ion of the alloying elements have a higher co-ordination capacity than the bulk metal ion, at least in the case of aluminium brass.

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