MICROBIALLY INFLUENCED CORROSION OF INDUSTRIAL MATERIALS

- BIOCORROSION NETWORK -

(Brite-Euram III Thematic Network N° ERB BRRT-CT98-5084

Biocorrosion 99-02

Meeting held on May 17-18 1999

Mülheim an der Ruhr, Germany

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FOREWORD

How an microorganisms influence corrosion ? This can be easily understand in principle, as the kinetic of corrosion depends upon the physico-chemical conditions at the interface, such as pH value, oxygen concentration, redox potential, water content and ionic strength. As the microorganisms adhere to the corroding surface, by means of their physiological activity they will be able to change all of these parameters in the most corrosion relevant way, i.e., directly at the interface. Thus, biofilms are the key to detecting, understanding, mitigating and preventing microbially influenced corrosion.

However, biofilms are a fairly new field in microbiology and not accessible to conventional microbiological methods. Suitable techniques for their investigation have been developed only recently, and the corrosion relevant biochemical and microbiological parametes first have to be identified.

Therefore, task 1 of the MIC network is dedicated to biofilms and their investigation. The first workshop was held in order to start the presentation and exchange of methods, ideas and interpretation of data of the participants.

The meeting was held with the support of the Institute for Water Chemistry and Water Technology (IWW) which is gratefully acknowledged. I also would like to thank the participants for their presentations and in particular for the creative and lively discussion, which happened in a very open and inspirative atmosphere. I also want to express my gratitude to BRITE EURAM for creating a network which allows such fruitful events.

Hans-Curt Flemming
Head of the Department of Microbiology of the IWW

LIST OF THE PRESENTATIONS

« Basic methods for biofilm characterization »
by HANS-CURT FLEMMING, IWW

« Isolation and characterisation of extracellular polymeric substances »
by THOMAS GRIEBE, UNIVERSITY OF DUISBURG

« Atomic force microscopy in biofilm research »
by ROLF GUBNER, PORTSMOUTH POLYTECHNIC

« FTIR-spectroscopy and time-of-flight-mass-spectrometry in biofilm investigation »
by ANDREW LEIS, UNIVERSITY OF DUISBURG

« Measurement of electron transport systems in biofilms »
by VITTORIA SCOTTO, UNIVERSITY OF GENOVA

« Microelectrodes »
by ALAIN BERGEL AND A. SAQUET, UPS-CNRS TOULOUSE

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SUMMARY

Microbially influenced corrosion (MIC) is a biofilm problem as the microbial influence is due to layers of microorganisms in close contact to the interface where the corrosion process takes place. Thus, reliable and representatie information about biofilms is mandatory for a better understanding of MIC and for the design of rational counterstrategies.
The purpose of task No. 1 is to provide and critically discuss methods for the investigation and possibly for the monitoring of biofilms and their development. The workshop on "Biofilms", held in Mülheim/Ruhr on May 17-18 1999, was dedicated to that task.

The question wether enzymes are involved in the corrosion process has been discussed and the contribution of V. Scotto strongly hints in the direction of enzymatical participation (see Scotto manuscript).
The basic methods for biofilm analysis are presented (see Flemming manuscript). However, the general problem is acknowledged that it is still not clear which biofilm parameter is actually related to MIC. Biofilm analysis provides coincidental evidence but not yet causal evidence, as there are many occasions in which biofilms are present without causing MIC.
Atomic force microscopy is a new and powerful tool for biofilm investigation (see Gubner & Beech manuscript). It allows the measurement of forces in the nanometer scale which can give further insight into the mechanisms underlying MIC.
It is generally acknowledged that the possibility to follow the process by means of monitoring devices is a primary demand in MIC investigation and mitigation. Electrochemical microsensors were presented for the analysis of both biofilms and electrochemical processes (see Saquet & Bergel manuscript).

Exchange of scientific personnel during year 1

1. Dr. Thomas Griebe from the University of Duisburg visits Prof. Vittoria from November 22 to 23 in order to provide biochemical methods as established at the IWW on the extraction of EPS as well as the isolation, characterisation and activity measurement of extracellular enzymes which are suspected to be involved in MIC processes. It is intended that after this first visit further visits will follow in order to establish the methods at the Laboratory of V. Scotto and to define further collaboration.

2. Dr. Andrew Leis from the University of Duisburg visits the Portsmouth laboratory from 13. - 23. of November in order to carry out measurements on microbial adhesion and cohesion forces by atomic force microscopy and to do corrosion measurements with corroded steel coupons which were incubated by a new microbial isolate from drinking water biofilms (so-called "Aquabacterium").

Further plans

The exchange of methods for biofilm and corrosion product investigation will be maintained throughout the entire period of the project.
The next workshop is planned for April 12-14 in Genova, Italy.

A focus for further work will be undoubtedly the isolation and analysis of EPS components, and in particular, exoenzymes. This will be a topic at the next general meeting of the MIC network,

represented by a lecure of HC Flemming on EPS. Another focus is the measurement of forces and currents within biofilms and between biofilms and substratum.
A third focus lies on the techniques for monitoring biofilms and/or MIC.

The exchange of scientific personnel has just started and will increase during year 2 significantly, as the links between the groups will be established.

In the appendix, the contents of the presentations are given in manuscripts of recent publications.

CONTRIBUTIONS

« Steps in biofilm sampling and characterisation in biofouling cases »
by G. Schaule, T. Griebe and H.-C. Flemming

« Microbiologically Influenced Corrosion of Industrial Materials »
by R. Gubner and I. Beech

« Relation between the biochemical structure of marine biofilms and the oxygen reduction kinetics on stainless steels »
by V. Scotto and M.E. Lai

« Electrochemical microsensors and microelectrodes : Applications in biofilm analysis »
by A. Saquet and A. Berge.

Steps in biofilm sampling and characterization in biofouling cases

Gabriela Schaule[1] , Thomas Griebe [2] and Hans-Curt Flemming [2]

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Introduction

If problems arise in technical water systems which are suspected to originate from biofouling, it is important to verify this assumption in order to design appropriate countermeasures. As biofouling is generally a biofilm problem (Characklis et al., 1990), detection of biofilms is the analytical key technique in order to verify the diagnosis "biofouling".

The first step in a rational anti fouling strategy frequently will be the analysis of deposits. In most cases, the deposits in technical water systems will not consist only of biomass but contain considerable amounts of abiotic inorganic and organic material, e.g., incrustations of mineral deposits, corrosion products or organic conditioning agents. The biological analysis verifies the presence of microorganisms and their proportion of the overall composition of the deposit.

In general, biofilms contain the following main components:

- water (up to > 90 %)
- EPS (up to 90% or organic matter)
- cells
- entrapped particles and precipitates
- sorbed ions and polar and apolar organic molecules

In some instances, a biofilm may accumulate lots of inorganic particles because of the adhesive properties of the EPS. These particles can contribute more to the overall mass of the deposit than the biofilm itself. In order to understand the fouling process, it is important in such cases to consider that the biofilm acts as a "glue" which causes the deposition of the material in the particular place. In fouling situations, the proportion of microorganisms can be even smaller, almost imperceivable within thick, crusty deposits. However, it is important to detect them, because they possibly play a crucial role in the formation of such deposits. An example is the excessive deposition of ferric oxide-hydroxyde by *Gallionella* sp., a case in which very few microorganisms and, consequently, a very small amount of biomass causes a large deposition of inorganic material. Thus, biofouling analysis in technical systems usually also has to deal with abiotic components. A typical example for the result of a deposit analysis is given in figure 1

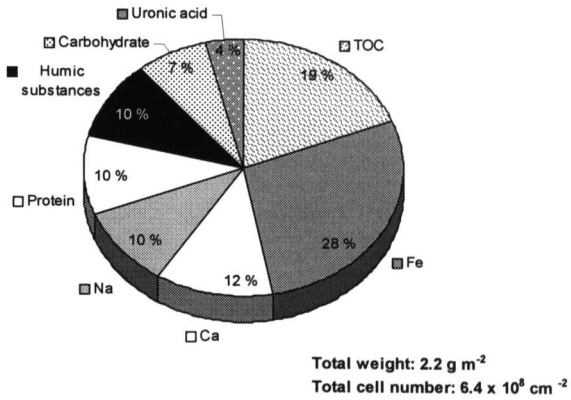


Figure 1: Composition of a deposit from a reverse osmosis membrane (Griebe, unpubl.)

Sampling strategy

The most common way to address biofouling problems is sampling of the water phase. This can usually be performed relatively easily but will provide no information about the location and the extent of deposits on surfaces. It is very important to keep in mind that microbial analysis of the water phase is not suitable to accurately locate or quantify biofilms as the contamination of the water phase occurs not continuously but randomly and does not reflect the site or extent of biofilms in a system. This emphasis on sampling of surfaces may sound trivial, however, in practice, surface sampling is often difficult.

In the first place, samples can be taken either by removing material directly from surfaces or by removing parts of the system carrying the fouling layers. This decision has to be taken at the site where the fouling problem occurs. In case the sample is taken directly, preliminary investigations must be carried out in order to design the most suitable protocol for effective removal of the material from the surface. The sampling surface area has to be determined. If it is necessary to remove parts of a system or, e.g., filter or ion exchanger material, it is mandatory to keep the samples under constant humidity, and transport should be performed cooled and mechanical shocks should be avoided.

In systems prone to biofouling it has proven very useful if surface parts are exposed which can be removed, commonly called "coupons". This facilitates the monitoring of biofilm accumulation. In drinking and process water systems, bypass monitoring systems are in use such as the biofilm monitoring device (van der Kooji et al., 1995), based on A TP measurement. Another option is the exposition of coupons under the hydrodynamic conditions of, e.g., the piping walls, known as "Robbins devices" (Ruseska et al., 1982). Exposition of surfaces in bypass systems are also used for monitoring. A suitable device is an annular reactor in which a cylinder rotates in a vessel, providing a defined shear field. The inner walls of the vessel carry specimens of materials on which biofilm accumulation is to be investigated; is called a prototype "RotoTorque" (Characklis, 1990) and has been modified in order to meet even more requirements in biofouling monitoring (Griebe and Flemming, 1996). In reverse osmosis membrane biofouling cases, sacrificial elements are used in a bypass, allowing access to representative surfaces (Winters et al., 1983; Ridgway et al., 1984). All these systems clearly have their merits, however, they require a good knowledge of the principle they are based on and a good deal of experience in order to interpret the results. Further developments are in process and there is still a strong demand for simple, accurate, non-destructive and real time monitoring.

Field methods in situ

Some simple field methods can give first clues about biofouling. In many cases, optical inspection can give a good indication of the presence of biofilms, and in some cases, characteristic smells confirms the diagnosis. Biofilms in technical systems have usually reached the plateau phase of growth and are in most cases visible to the bare eye. In technical systems, they tend to display a slimy consistency which can be detected by wiping; thin layers can be made visible if a white tissue is used for wiping. In case of doubt about the biological origin of a deposit or layer, it is useful to take a small amount of the material and put it over a lighter until it smolders. A smell of burnt protein is characteristic for biological material.

Laboratory methods

In many cases, field methods are not sufficient in order to define biofouling and laboratory investigations are required. A wide range of methods is available fur this purpose and the selection of a particular method depends essentially on the question to be answered, e.g.: is the biofilm is hygienically relevant? Does it block filters or membranes? Does it contaminate ultrapure water?

Also, the substratum with it's surface texture, chemistry and geometry and the nature of the deposit have to be considered. A first differentiation is possible by microscopy, followed by biochemical methods. While all methods mentioned in this paper are valid for biofilms removed from their substratum, only a few of them are applicable for direct analysis of the non disturbed sample. The latter are discussed in the following paragraphs.

Methods directly to examine adherent microorganisms

Microscopy

The most common method for enumeration and morphological observation of microorganisms on surfaces is microscopy. This includes direct counting methods such light microscopy, epifluorecene as microscopy, scanning electron microscopy (SEM), Confocal Laser Scanning Microscopy (CLSM). If the biofilm is thin, direct epifluorescence microscopy will be the most feasible method, using autorluorescence staining with 4,6-diamidino-2-phenylindole (DAPI) or acridin orange (AO) as DNA specific dyes, indicating the total number and distribution of cells. Biofilms of more than 3-4 µm thickness usually cannot be handled with common light microscopes because material above and below the focal plane will scatter light and interfere with the direct measurement. Such biofilms can be investigated non-destructively either by computer enhanced microscopy (Walker and Keevil, 1994) or by CLSM (Lawrence et al., 1991; Caldwell et al., 1992). The CLSM allows optical sectioning of the structure of the biofilms. With image analysis, the three dimensional reconstruction of the undisturbed sample is possible. This is of particular interest for, e.g., biocorrosion studies.

Direct enumerating methods have to take into account the possible interference of the substratum and it may be necessary to remove the microorganisms to be able to enumerate or to characterize them. Polymer materials such osmosis membranes are often as reverse autofluorescent over a wide spectral range and fluorescent dyes cannot be applied. Therefore, removal, homogenization, dilution or concentration by membrane filtration may be required prior to enumeration.

Scanning electron microscopy (SEM) provides another option for the investigation of biofilms. However, it must be taken into account that the process of sample preparation includes complete dewatering. Considering that biofilms consist mainly of water, the picture obtained is necessarily an artifact. It still gives insight into the structure of the biofilm and can reveal the presence of different

kinds of extracellular polymer substances (EPS). In order to avoid excessive shrinking of the sample, the EPS are stabilized with Ruthenium red and dewatering can be performed, using a fluorocarbon compound which allowes drying by sublimation (Griebe and Flemming, 1996). A new SEM technique is now available which allows overcoming this obstacle. It is called "Environmental Scanning Electron Microscopy" (ESEM). With this method it could be demonstrated that the sample preparation process for classical SEM leads to a considerable loss of material (Little et al., 1996). However, the magnification achieved by ESEM is much lower than with the conventional SEM.

Nucleic acid specific dyes

DNA and RNA provide large numbers of intracellular binding sites that marked fluorescence promote enhancement of many stains such as DAPI, AO, Hoechst 33258, 33342, SYTOX Green, SYBR green, (Weinbrenner, 1998), PicoGreen and propidium iodide. (Roth et al., 1997). Their application is supposed to allow to detect the organisms in a deposit and to distinguish them from particles.

Vitality dyes

The vitality of the microorganisms in the biofilm can provide a very important information, e.g., in order to assess the efficacy of biocides. This can be performed using vitality dyes. They allow the recognition and localization of physiologically active bacteria in biofilms. A prerequisite is a biochemically induced reaction within the cell. If a fluorescent dye is used, the reaction can be observed with incident light using an epifluorescence microscope or a CLSM. An example is the reduction of the colourless, soluble 5-cyano-2,3-ditolyl water tetrazolium salt (CTC) to an insoluble and fluorescent formazan by the cellular respiration chain, which is fluorescing in red after exitation (Rodriguez et al., 1992; Schaule et al., 1993). Further examples are other fluorogenic substrates such as sulfofluoresceine diacetate (SFOA) or carboxyfluoresceine diacetate (CFOA, Brul et al., 1997). Enzymatic activity leads to a hydrolytical cleavage of substrate and a fluorochrome which fluoresces after exitation.

Apart from the determination of intracellular enzymatic activity and redox potential, the integrity of the cell membrane is generally considered as an indicator of the viability of bacterial cells. Probes which detect the membrane integrity include positively charged fluorochromes such RH- 795, Carbocyanine as derivatives and Rhodamin 123 (Morgan et al., 1993) or the exclusion of negatively charged bis-oxonols by active and intact bacteria (Jepras et al., 1995; Lopez et al., 1996; Mason et al., 1994, 1995). The most rapid and BacLightR, commonly used kit (Live/Dead test Molecular Probes) for the assessment of viability is based on the staining with the membrane potential sensitive dye propidium iodide and the nucleic acid stain SYTO 9 (Roth et al., 1997). Lawrence et al. (1996) reported problems associated with the use of this test kit when applied to biofilms. The green nucleic acid stain SYTO 9 often failed to penetrate microcolonies of living cells that were embedded in EPS which led to an underestimation of the number of viable cells.

Antibiotics such as nalidixic or pipemidic acid prevent cell division and allow to detect living bacteria by their elongation after incubation with substrate and staining with a fluorochrome (Kogure et al., 1979; Yu et al., 1993).

Reduction activity

Dehydrogenase activity can be determined by the use of tetrazolium dyes. Examples are triphenyl tetrazolium chloride(TTC,Ryssov-Nielsen,1975), 2-(p - biodophenyl) - 3 - (p - nitrophenyl) - 5 phenyl tetrazolium chloride (INT, Chung and Neethling, 1989) and CTC (Rodriguez et al., 1992; Schaule et al., 1993). The usual procedure is to incubate the sample with the reduction dyes, to extract the formazans after the reaction time and to determine the overall fluorescence intensity. In presence of elevated levels of non-biological material or in thick biofilms, INT and TTC are less suitable than CTC in order to indicate microbial redox activity because they form non fluorescent formazanes in contrary to CTC with the consequence that the crystals cannot be visualized by incident light and, thus, not be detected in biofilms directly on surfaces. In strongly reduced samples, the redox potential can be as low as -200 mY with the consequence that INT and TTC are reduced abiotically to formazan while CTC is reduced only from 220 mY (Seidler, 1991). Another useful redox dye is resorufin which has been used as an indicator of cytoplasmatic reducing potential in yeast cells (Caldwell et al., 1992).

Biofilm thickness

The thickness of a biofilm or a fouling layer can be of interest, e.g., in cases of membrane fouling in RO processes. If the layer is sufficiently transparent, it is possible to assess the thickness by microscopical methods (Bakke and Olson, 1996). , but in most cases, the layer will be opaque and of grey, reddish or brownish colour. In such cases, thickness determination by cryosectioning can be an interesting alternative (Yu et al., 1993). An example is the investigation of fouling layers on reverse osmosis membranes (Griebe and Flemming, 1986). The steps are schematically depicted in figure 2.

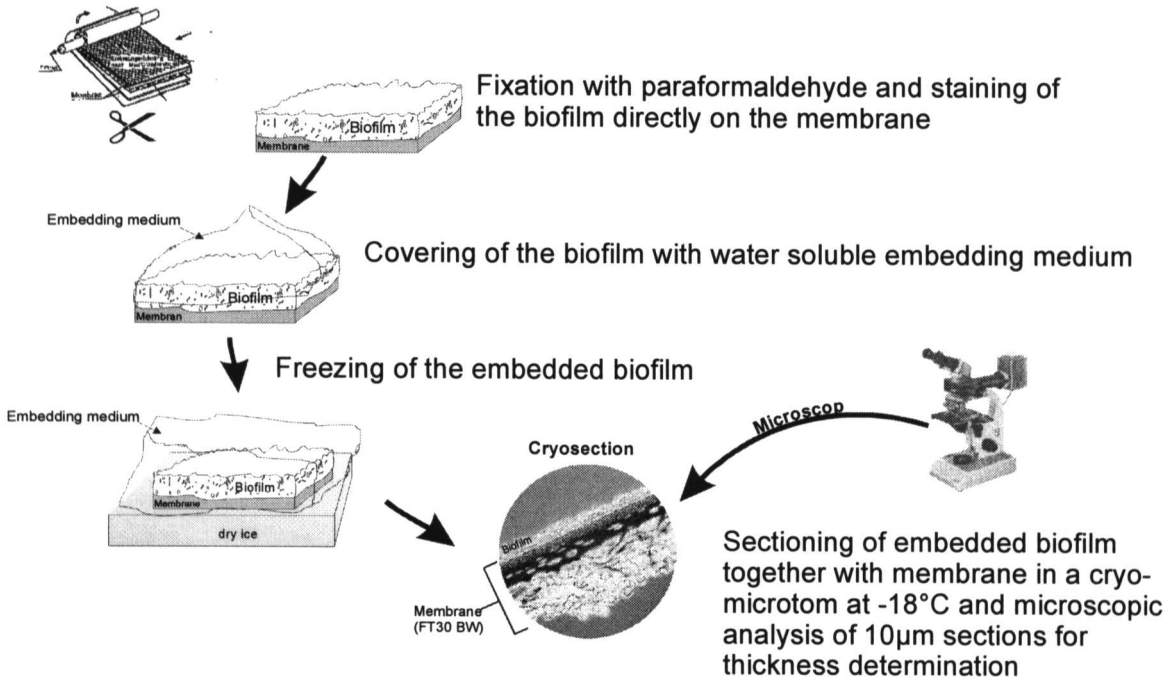


Figure 2: Steps in cryosectioning for the determination ofbiofilm thickness and structure in biofouling analysis on reverse osmosis membranes

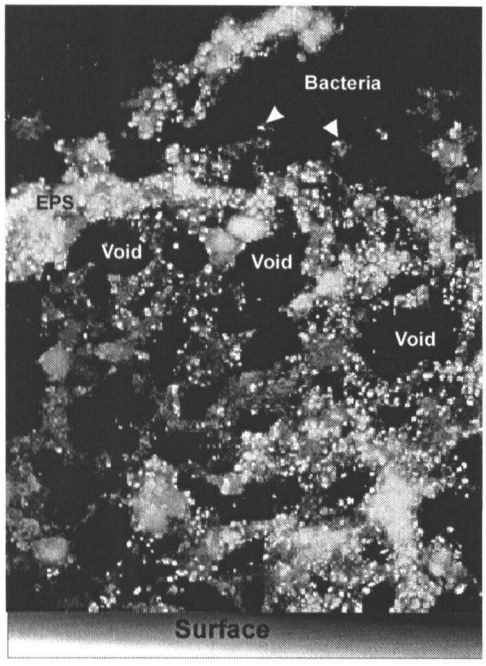


Figure 3: Cryosection of an environmental biofilm. A thin cross-section (0,5 µm thickness) enables the differentiation of single bacteria (AO stained) in clusters, pores (labelled P) and EPS (arrow) stained with FITC (Griebe, unpubl. observ.)

In special cases, mostly for research purposes, it can be of importance to elucidate structure and composition of biofilms in further detail. Detailed information about microcolony cluster size, porosity, voids and cell density can be acquired by using specific stains and staining techniques in combination with cryosectioning (fig. 3).

FTJR-ATR spectroscopy for characterisation the composition

FTIR spectroscopy has been used in biofilm investigation since quite some time (Nivens et al., 1985, Schmitt and Flemming, 1996) and is particularly useful if biotic and abiotic components occuring in a deposit are to be differentiated. A lot of practical experience was gained by the investigation of fouled membranes; in many cases biological and other kinds of fouling usually coincide an have to be differentiated (Flemming et al., 1998). In order to do so, FTIR spectroscopy is applied in the attenuated total reflexion (ATR) mode. This technique allows the analysis of smooth surfaces directly. Practically no sample preparation is required. The acquired absorption reveal the chemical spectra composition of the deposit. Biological material is detected by the occurrence of the typical amide I and II bands which originate from proteins (Schmitt and Flemming, 1998).

Methods to remove microorganisms from surfaces

Mechanical force

Mechanical removal of biofouling layers includes e.g., scraping with various tools such as scrapers, flat knifes or abrasive removal using glass beads or other particulate material. In order to remove and homogenize thick layers, often a combination of different scraping methods followed by ultrasonic treatment is feasible.

Biofilm detection by direct swap collection is one of the few standardized methods to remove thin biofilms. A defined area of the wet surface is swapped with a polypropylene swap. The collected material is transferred into a bottle of sterile water and analyzed. The method is validated and documented in the *Sematech Provisional Test Method for Determining the Surface Associated Biofilms of UPW Distribution Systems 920J0958B-STD (1992)* This method is applicable for smooth system surfaces as well as for parts which can be removed from the system. It is not feasible for pipes with small diameter, or rough inner surface or if areas in a long distance to the access point have to be sampled. In such cases, other methods such as the use of glass beads with a smooth surface and approximately 0,5 mm in diameter can be used for abrasion. Schaule and Flemming (1997) first introduced this method for ultra pure water systems.

An Example: Biofouling in a ultra pure water (UPW) system

The irregular occurrence of high numbers of colony forming units in the water phase indicates the presence of a biofilm contaminating an UPW system. In order to locate the contamination source, it is recommended to divide the system into different sections from which water samples are taken These samples are analyzed microscopically for the presence of flocs which are indicative for biofilms. Such flocs are not detected by cultivation methods with subsequent plate counting. With this strategy, the most contaminated section of the system can be identified. Then, parts of the section situated in front of that area are removed (e.g., pipes), and the fouling layer is completely removed using the following protocol:

1. One end of the pipe is closed aseptically. A defined volume of sterile water and sterile glass beads are added
2. The other end is closed aseptically. The pipe is carefully shaken for several minutes and turned around the long axis in order to allow contact of the glass beads to the entire inner surface
3. One end is opened, the glass beads and the water are collected in a sterile bottle
4. The supernatant is separated after approximately one minute and analyzed for any interesting parameter

Ultrasonic treatment

Ultrasonic treatment is a widely used and reliable method to remove and disaggregate biofilms. To avoid cell lysis, usually sonication time of 1 to 3 minutes is chosen and with commercially available sonication baths, a frequency of 40-50 Khz, best results are obtained in terms of removal and survival of cells. It should also be taken into account that the position of the sample relative to the sonicator can change the effect significantly (Zips et al., 1990). This is one of the results of a round robin test carried out by an European task group for evaluation of the removal of biofilms. The results of the round robin tests showed that 3 minutes of sonication was in most cases sufficient for effective biofilm removal. Nevertheless it should be noted that the yield was strongly dependent of frequency, energy, distance to the sample and geometry of the sonification device.

Laboratory methods to examine removed biofilms

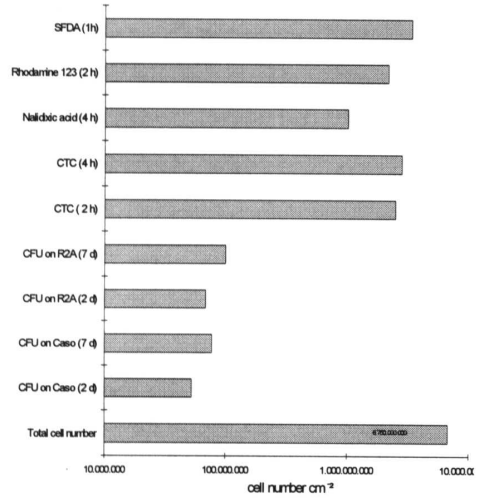
Methods requiring cultivation

Classical microbiological identification methods are based on cultivation of the organisms. Since all media are selective to a lesser or greater extent and not all bacteria or other microrganisms are culturable, viable counts rarely represent all microorganisms present in a sample. Therefore, it is useful to determine the total cell number by methods as described earlier. This number allows estimating the ratio of culturable cells.

Colony forming units (CFU) and Most Probable Number (MPN) method

CFU plate counting is the most commonly used method to monitor water quality. There are standardized methods for drinking water, ultra pure water, water for injection, waste water documented (e.g., national legal regulations and pharmacopoeae). In biofouling cases, it has proven useful to expand the range of media and incubation parameters in order to apply the most sensitive method. For better comparability it is generally recommended to use R2A medium (Reasoner and Geldreich, 1985) and an incubation temperature of 20°C and 28 oC as defined in the ASTM. The results should be documented as CFU at 20°C after 7 d in order to include organisms, which are not detected after shorter incubation times. The application of cultivation methods to homogenized biofilms can hold certain disadvantages. If the proportion of non-biological components of the deposit is high the membrane filtration as a method

for concentrating cells is of limited value. In such cases, the most probable number (MPN) method is useful. This is a standard procedure of classical microbiology. When assessing, e.g., the presence of *E. coli,* the number is not related to a volume of 100 ml but to a defined surface area or a defined quantity of the deposit. In a case study, various methods for the determination of viable and vital bacteria from a reverse osmosis (RO) membrane biofilm were compared (figure 4). This example clearly demonstrates the differences between plate counting technique and microscopic techniques for the detection of vital bacteria.



Parameter and detection	Percent of total cell number
Hydrolytic (esterase) activity (microscopic detection)	50
Membrane permeability (microscopic detection)	32
Elongation (gyrase activity) (microscopic detection)	15
Dehydrogenase activity (microscopic detection)	41
Dehydrogenase activity (microscopic detection)	37
Cultivation on low nutrient medium	1,5
Cultivation on low nutrient medium	1,0
Cultivation on nutrient rich medium	1,2
Cultivation on nutrient rich medium	0,8
Nucleic acid marker (microscopic detection)	100

Figure 4: Comparison of data obtained from a RO-membrane biofilm sample by microscopic and cultivation methods for the determination of total and viable bacteria. The preparation procedure and incubation time and the cell numbers are shown (Griebe, unpubl.).

Identification

Identification by analysis of phospholipidfatty acids

The analysis of the phospholipid fatty acid (PLF A) profile has been developed for the characterization of microbial populations in soil and sediment samples. It is based on the composition of the phospholipids that are present in the membranes of all living cells. They can be extracted and quantified very sensitively (Tunlid and White, 1990). This method allows the assessment of the quantity of biomass at a given site. Phospholipids belong to the first compounds to be degraded when cells are decaying (within hours), therefore, the content of PLF A indicates the amount of living biomass. As the composition of fatty acids is different in different organisms, the PLF A profile can be used in order to analyze the composition of the population (White, 1993; Zelles and Bai, 1994) and is used as taxonomical marker (LeChevallier, 1977; Vestal and White, 1989).

An advantage of the method is that the samples can be analyzed directly and without prior treatment. However, it is mainly suitable for relatively high concentrations of biomass as encountered in soil and sediment samples while populations of thin biofilms will be difficult to analyze with that method. Comparison with other methods such as the content of ATP, DNA and protein revealed a good correlation (White, 1988). The qualitative and quantitative determination of PLF A is performed by gas chromatography or gaschromatography/ mass spectrometry. The method provides the following information:

- Quantity of living biomass (Tunlid and White, 1990)
- Gross characterization of population composition (diversity of population, dominance of certain groups) (Tunlid and
- White, 1990)
- Nutrient situation (Kieft et al., 1994)
- Presence of toxic or inhibiting substances (Napolitano et al., 1994)

FT -IR Spectroscopy

A more rapid method to identify isolates is given by FTIR spectroscopy. Most structural and functional groups of different bacteria are identical and give the same signals. Thus, spectra of bacteria look very much alike. However, the quantity and distribution of the different groups vary significantly among microbial strains and this is where all approaches to the characterization of bacteria by their IR spectra are based on. The advantages of the modern FTIR spectrometers, the software and elaborated mathematical and statistical algorithms for analyzing IR-spectra allows the detection of the differences well enough in order to distinguish different bacterial strains,

and identify them, if there are references available (Schmitt and Flemming, 1995). A considerable library of reference spectra for drinking water bacteria has been established. The occurrence of bacterial storage products as polyhydroxy-alcanoates such as polyhydroxy butyrate (PHB) or other biopolymers can be detected in FTIR spectroscopy as well. This allows the observation of physiological reactions of microorganisms, since these substances are formed under and unbalanced nutritional stress conditions.

Chemical and biochemical analysis

Water content

The determination of water occurs by measurement of the sample before and after drying at 105°C to a constant weight. In most cases, it will be necessary to remove them from the surface in question in order to determine the weight. The exact quantification of the water content can be difficult in cases when thin biofilms are exposed to the atmosphere and the water evaporates quickly. In such cases, it may be useful to remove the fouling layer under 100% humidity.

Total organic carbon (TOC).

The total organic carbon (TOC) content gives an idea about the proportion of organic matter in a sample and can be interpreted as biological material, e.g., in corrosion products in which no other organic material is to be expected. The chemical and biochemical analysis is one approach to measure the biomass of biofilms. A number of intra and extracellular components, such as total organic carbon (TOC), protein, lipopolysaccharide, carbohydrate or muramic acid content have been successfully used to quantify universally distributed biofilm components. In table I, some parameters indicative for the presence of biofilms are summarized.

Table I: Examples for biofilm parameters

Parameter	Detection method [reference]
Water content	24 h, 105°C
Organic carbon	TOC, COO, incineration loss
Protein	[Lowry et al., 1951 ; Bradford, 1976
Carbohydrates	[Dubois et al., 1956; Raunkjaer, 1994]
Uronic acids	[Blumenkranz and Asboe-Hansen, 1973]
DNA	[Palmgren and Nielsen, 1996]
Lipids	[Geesey and White, 1990]
Muramic acid	[Geesey and White, 1990]
Polyhydroxybutyrate	[Geesey and White, 1990]
Total cell number	[Hobbie et al., 1977]
Colony forming units	various standard methods
ATP	[Karl, 1993]
Hydrolase activity	[Obst and Holzapfel-Pschorn, 1990]
Respiratory activity	[Schaule et al., 1993]
Indolacetic acetic acid production	[Bric et al., 1991]
Catalase activity	[Line, 1983]

Polysaccharides and Proteins

Calcofluor white (Wood, 1980), Primulin (Caron, 1983) and conjugated lectins (Wolfaardt et al., 1998; Caldwell et al., 1992) bind to polysaccharides. Lectins which bind to specific combinations of sugar molecules have been used for the identification and localization of cell types (e.g.. gram positive or negative) or components in the matrix of EPS (Lawrence et al., 1994). A serious pitfall of the uncritical use of lectins such as concanavalin A (Con A) and wheat germ agglutinin (WGA) which are conjugated with flurescence dyes -such as fluorescein isothiocyanate (FITC)- is their non specific binding potential to proteins (Babuik and Paul, 1969; Hara and Tanou, 1989). This leads sometimes to an accumulation of lectins in the EPS matrix of biofilms. In recent publications, lectins are coupled to different fluorochromes (TRITC, AMCA-S, Oregon Green, tetramethylrohodamine and Texas Red)(all available from Molecular Probes; www.probes.com). This allows the application of more than one lectin at a time in order to selectively label and map different sugar molecules because the signals can be differentiated by their specific fluorescence emission. The determination of the content of protein (Lowry et al., 1951; Bradford, 1976) and carbohydrates (Dubois et al.,1956; Dreywood, 1946) can also be used to roughly assess the content of biomass. These parameters are of particular interest if the samples contain a high proportion of non-biological material, e.g., sediments, soils, corrosion products etc. However, interference with humic substances can lead to erroneous results and may require sample adequate modifications of the methodology as done for the above cited parameters by Raunkjær et al. (1994) and Frølund et al., 1995.

Adenylate content

The content of ATP or the overall adenylate content can be used to quantify the biological activity in a biofilm. In order to do so, the adenylates are extracted from the sample and subsequently quantified by bioluminescence (luciferin/luciferase system) by HPLC chromatography (Karl, 1993). The ATP content depends upon the state of activity of the organisms in the biomass. It must be taken into account that transport and storage of the sample is of strong influence to the physiological activity of the microorganisms (Griebe et al., 1997). Thus, sampling and handling details can influence the data very much. Nevertheless, the determination of the ATP content belongs to the common methods in biofilm research (Geesey and White, 1990).

Hydolytical activity

Independent of the redox potential, microbial activity can be determined by methylumbelliferon or methyl-coumarinylamide substrates, indicating the activity of hydrolytical enzymes such as glucosidases, lipases and aminopeptidases. A correlation, however, of enzymatical activity and the number of sessile microorganisms or the overall biomass is not yet possible. A considerable proportion of the hydrolytical enzymes is located in the EPS matrix (Frølund et al., 1995) and it is not known for how long they are active after their mother cells may have died.

Genetical methods

Polymerase chain reaction (PCR)

Basing on the polymerase chain reaction (PCR), various analytical techniques can be used in order to identify biofilm organisms without prior cultivation. First, DNA fragments have to be extracted from the sample. It may be suitable to remove the organisms, e.g., by application of ultrasonic energy, then they are concentrated by membrane filtration and lysed afterwards (Holben et al., 1988). It is also possible to lyse the cells directly in the sample and extract the DNA by ultracentrifugation (Steffan et al., 1988; Ogram et al., 1988). The use of polyvinylpolypyrrolidone is recommended in order toremove humic substances if these are present in the sample and can interfere with the PCR reaction. After amplification of the extracted rDNA a mixture of amplification products is obtained which has to be separated by molecular biological techniques and cloned subsequently. Restriction analysis of the cloned rDNA fragments is performed and the pattern of the fragment analysis can be compared to that of the cultivable organisms of the population. This way it is possible to obtain genetical fingerprints of bacteria, protozoa, fungi, metazoa and algae possibly present in the sample. An efficient analysis of the microorganisms by means of PCR fingerprinting can be supported by using DNA sequencers, capillary electrophoresis, image processing and data bases with reference patterns for the identification of unknown isolates (Tichy et al., 1996).

Gene probes

With rRNA directed and fluorescence labelled oligo nucleotide probes it is possible to detect and localize microorganisms which do not necessarily have to be culturable and to locate them in the phylogenetical tree (Alfreider et al., 1996; Ogram and Sayler, 1988; Wagner and Amann, 1996). More than one oligonucleotide can be applied in order to label more than one kind of organisms. This method provides not only information about the species actually present in the undisturbed sample but also reveals their position in relation to each other, using the potential of the CLSM to demonstrate three dimensional structures. This makes it particularly suitable to investigate the functional architecture of a biofilm. In addition, the intensity of the signal depends upon the ribosome content and, thus, reflects the physiological status. In order to apply the gene probes, the cells must become permeable. The procedure required for that purpose will kill them and it cannot be completely excluded that the architecture of the biofilm is untouched by the procedure. Nevertheless, this method belongs to the most important progress in biofilm research and is, therefore, separately acknowledged in the chapter of Kalmbach et al. and Schwartz et al. in this volume.

Immunological methods

Detection and identification of biofilm population members can also be performed by immunological coupling reactions between fluorescent labeled antibodies which are specific to certain organisms (Gaylarde and Cook, 1990; Griffin and Antloga, 1985). This method requires the cultivation of the target organisms and the subsequent production of antibodies and is suitable to detect them in a thin biofilm (Hausner et al., 1996 this volume). In multilayered biofilms, the EPS matrix acts as a diffusion barrier for large molecules and the antibodies will take some time to reach the antigenic sites of the target organisms. The advantage in the use of mono- and polyclonal antibodies is that the cells are not killed by this method and, thus, processes in the biofilm still can be observed. Also, the combination with other non-destructive fluorescence staining methods is possible, e.g., with CTC.

Conclusions and outlook

The investigation of deposits on surfaces, in particular of biofilms, represents a challenge for analytical chemistry and microbiology. Although it is possible to detect very low concentrations of

chemicals or microorganisms in a given volume of water or gas, the options for the detection of deposits is still somewhat dissatisfying. Until now, it is necessary to remove the material from the surface and investigate it subsequently. Some of the methods which apply specifically to biological material have been presented in this chapter. All these methods are more or less time consuming and yield results usually only hours or even days after sampling - this is the actual state of the art. Although the analysis of surfaces in the scale of a few Angstrom or nanometers is very well developed, the analysis of surfaces in the range of square centimeters or meters is still dependent on scratching material from the surfaces and subsequent analysis in the laboratory.

New approaches to analyze biofilms and other deposits should provide qualitative and quantitative information on line, in situ, in real time and non destructively. Two strategies seem to be feasible to fulfill these demands.

i) Sensors which are installed at representative sites in a system, e.g., in a heat cooling water cycle, at a ship bottom or in a pipe line. A suitable approach is the use of fiber optical sensors or differential turbidity measurement (Flemming et al., 1998), and

ii) Probe heads which allow to scan surfaces in question, revealing information about contamination and deposits. This might be performed by the use of FTIR-ATR spectroscopy probe heads.

Such approaches will open a completely new field of surface analysis and in particular, to detection and monitoring of biofouling.

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The Potential Use of Atomic Force Microscopy for Studying Corrosion of Metals in the Presence of Bacterial Biofilms-an Overview

Rolf Gubner and Iwona B. Beech

ABSTRACT

This communication outlines the principles of application of scanning probe microscopy (SPM) as a tool for studying physico-chemical and biological phenomena and discusses the potential use of atomic force microscopy (AFM), a form of SPM, for investigation of bacterial biofilms formed on metal surfaces and for studying corrosion of these surfaces in the presence of such biofilms. AFM images showing biofilms developed in pure cultures of either Pseudomonas *species on copper, or by a marine isolate of sulphate-reducing bacterium on stainless steel 304, are presented to demonstrate usefulness of the SPM technique for both quantitative and qualitative determination of biocorrosion.*

METHODS OF MICROSCOPY USED FOR STUDYING BIOCORROSION

The role of biofilms in the deterioration of metals and their alloys in both fresh water and marine environments has been of considerable interest to industry worldwide. Problems associated with biocorrosion due to the formation of biofilms are widespread and have serious economic and safety implications (Hamilton, 1985). However, to date it still remains to be elucidated how much a role in the corrosion of metals does microbially influenced corrosion (MIC) play and what are the key mechanisms involved.

Methods frequently used for microscopic investigations of biofilms and MIC include techniques of scanning electron microscopy (SEM), (Beech, 1991), environmental SEM (ESEM) (Wagner et al., 1992) and different forms of light microscopy (Caldwell et al., 1992; Keevil & Walker, 1992). Although useful, these techniques have their drawbacks. SEM has the disadvantage of a lengthy sample preparation and sample distortion due to the requirement for dehydration. Light microscopy overcomes the problem of specimen shrinkage but does neither have the resolving power nor the magnification that SEM offers. ESEM techniques need some improvement in order to achieve the image resolution required for detailed observation of biofilms in their fully hydrated form.

Conventional microscopy techniques do not allow real-time visualisation of the surface of a bacterial cell or of the events involved in MIC such as initiation of pitting or synthesis of extracellular polymeric substances (EPS). Methods, which permit *in-vivo* observation of bacterial biofilms on metallic and non-metallic surfaces such as differential interference contrast and confocal microscopes, lack resolution to simultaneously image individual cells and the substratum.

Recently, researchers investigating MIC have been exploring one of the scanning probe methods (SPM), Atomic Force Microscopy (AFM), to gain a better understanding of the true nature of bacterial biofilms developed on metal surfaces and to elucidate the involvement of these microorganisms in corrosion.

SCANNING PROBE METHODS

The general term Scanning Probe Methods refers to the class of microscopes that obtain images by scanning surfaces; these include Atomic Force Microscopes (AFMs), Scanning Tunnelling Microscopes (STM) and most recently developed Scanning Near Field Optical Microscope (SNOM, or NOSM).

The SPM offers high resolution, three dimensional magnification and operation in diverse environments. Applications of SPM are numerous and include the following:

- Imaging

(a) **Atomic resolution** imaging, for study of the atomic and crystalline structures of materials (metals, minerals, oxides, thin films) and corrosion reactions.

(b) **Molecular resolution** imaging, for study of biomolecules such as DNA and proteins, liquid crystals and polymer structures (size, uniformity, lamella).

(c) **Nanometre resolution** imaging, for study of man-made structures such as semiconductor devices and precision gratings and natural, biological structures such as cells and cellular components, viruses and antigen/antibody complexes.

- Measurements of small forces

(a) **Magnetic Forces** on magnetic or superconductive surfaces

(b) **Friction Forces** by simultaneous measurements of normal and lateral forces

(c) **Adhesion Forces** in the field of molecular recognition

- Manipulations

In addition to visualisation of a surface, the SPM allows a nanoscale manipulation of the surface as the process of imaging itself exerts a force on the sample. Atoms and molecules can be manipulated on surfaces (Xe on Ni; Fe on Cu and CO on Pt). Molecules that are loosely held can be moved around or swept off. The surface can be scratched to create submicroscopic indentations (eg. creation of nanostructure in a film of polystyrene and other polymers). Nanoscale manipulation offers a selective

manipulation of the matter at the molecular level by breaking of molecular bonds (i.e. a purposeful cleavage of a plasmid DNA at a specific site).

An important aspect of the SPM imaging is the ability to follow a molecular process in real time as environmental conditions are changed (eg. study of polymerisation of blood protein, fibrin upon addition of the clotting agent to a solution of fibrinogen, ageing studies by the investigation of film formation and annealing of polybutylmethacrylate latex cast from solution, solvent induced crystallization of polycarbon thin films, the exocytosis of a poxy virus from a living cell in a liquid environment and observation of a degrading polymer surface in a controlled pH environment).

Examples of the use of SPM in various areas of research are numerous, ranging from material science, corrosion, electrochemistry, organic and inorganic chemistry and biological systems (Gould et al., 1990). SPM imaging has been performed in a variety of environments including air, inert gas, liquid and high vacuum/ultra high vacuum.

Scanning Tunnelling Microscopy

The STM is capable of imaging at the Ångström scale, allowing the examination of the surface of conductors with atomic resolution. An image is obtained by measuring the motion of a sharp metal tip as it is scanned in the X-Y plane over a surface (Fig. 1). The gap between the tip and the sample is kept constant, (8 Å) with a feedback electronic circuit.

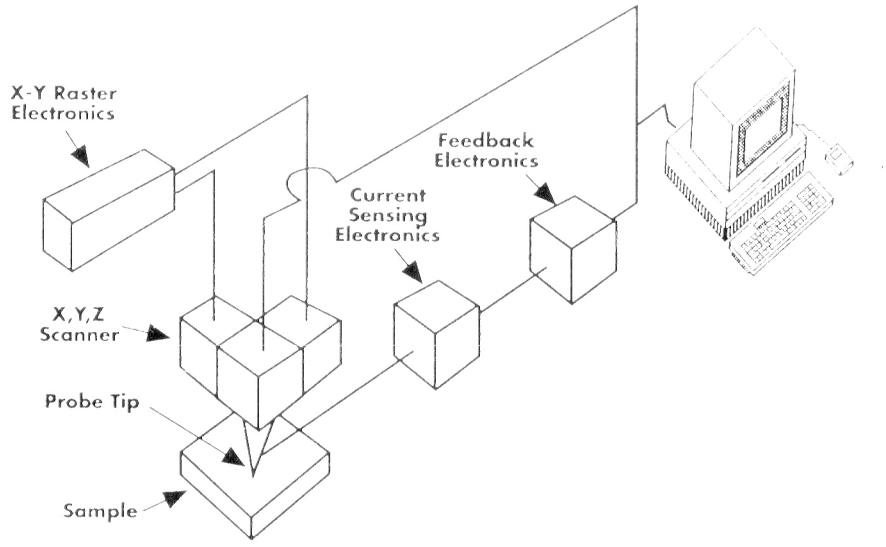

Figure 1: Components of an STM are an x, y, z piezoelectric scanner, a sensor for measuring the tunnelling current, feedback electronics for controlling the z piezoelectric ceramic, electronics for rastering the x and y piezoelectric ceramics, and a computer for displaying the images

The small motions required to control the tip in the x, y, and z directions are made using piezoelectric ceramics. A signal is generated due to the electron tunnelling between the probe tip and the sample surface. The tunnelling current is exponentially

dependent on distance. If the probe to sample gap changes by 1 Å, the tunnelling current changes by an order of magnitude. The vertical resolution of the STM can reach 0.1 Å.

Scanning Near Field Optical Microscopy

A sub-wavelength sized light source is scanned very close (within 10 nm) to a sample. A feedback mechanism is used to keep the aperture close to the sample and an optical image of the specimen is built pixel-by-pixel. The light source is an optical aperture (20-300 nm) fabricated by heat-drawing an optical fibre down to a fin tip. An opaque material, such as aluminium, is evaporated onto the fibre leaving a small aperture at the tip. The shape of this fibre is of a paramount importance to the quality of the image. Only an area of the size of the aperture is illuminated. A large collection lens detects the variations in light intensity as the probe is scanned across the sample, providing image data.

Atomic Force Microscopy

The AFM was invented in 1986 to visualise surface features of non-conductors on a molecular scale (eg. 30 Å on a lateral resolution and 1 Å on a vertical resolution). The AFM operates by measuring the forces between the probe and sample in order to generate images of the sample surface. These forces depend on:

- the nature of the sample
- the distance between probe and sample
- the probe geometry
- contamination of the sample surface

An AFM sensor consists of a cantilever with a probe (tip) mounted on it (Fig. 2). Most cantilevers are made from silicon nitride with lengths of 100-200 microns, thickness of about 1 micron and spring constant of 1N/m or less. The standard tips on these cantilevers are pyramidal with sides of about 4 microns, aspect ratio 1:1 and a dull end. Recently a new generation of tips has been developed with aspect ratios of at least 10:1.

As the probe is brought close to the sample, it is first attracted to the sample surface. A variety of long range attractive forces, such as van der Waals forces, are at work. When the probe gets very close to the surface, the electron orbitals of the atoms on the surface of the probe and sample start to repeal each other. As the gap decreases, the repulsive forces neutralise the attractive forces, and then become dominant. An image is created as the cantilever/probe is moved across the surface in a raster pattern. Both vertical deflections and torsion of the cantilever are detected by a laser system, which applies feedback to a piezoelectric ceramic that controls the distance between the sensor and the sample. The movement of the sample is used to build a three

dimensional image of surface topography. Topography and friction can be imaged simultaneously. The forces between the probe and surface can be as small as 1 nanoNewton; similar to the force between two neon atoms in close proximity.

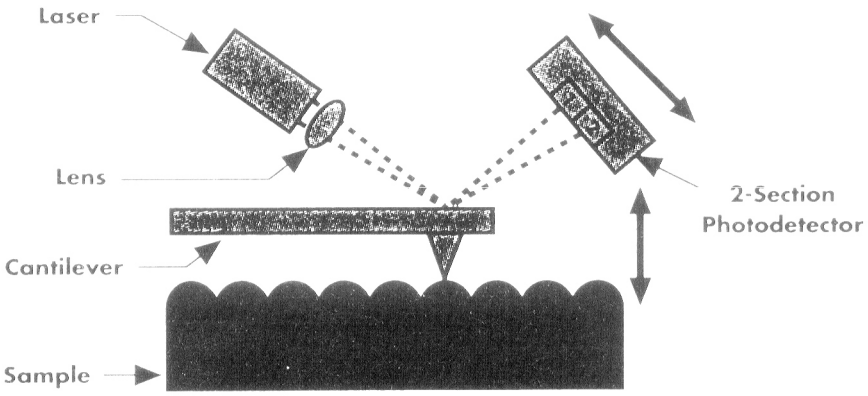

Figure 2: The sensor in the AFM uses a laser beam that deflects off a small cantilever onto a multi-section photodetector. When the cantilever moves up and down, the amount of light hitting each photodetector section changes. This provides a feedback signal for controlling the piezoelectric ceramic.

TYPES AND MODES OF OPERATION OF AFM

AFM Imaging modes can be classified as "contact" and "non-contact" depending on the net force between the probe and the sample.

Non-Contact AFM

Probe operates in the attractive force region. The cantilever is curved toward the sample. The tip is oscillated above the surface and measures the change in amplitude, phase or frequency of the oscillating cantilever in response to force gradients from the sample (Fig. 3a). In non-contact modes, images may be made of very soft surfaces that are not well adhered to their substrates.

Contact AFM

This is the standard mode of AFM imaging. Probe operates in the repulsive force region. Cantilever deflects as the tip encounters variations in surface topography (Fig. 3b). This mode is very effective when imaging submerged samples and particularly useful with soft samples.

Tapping Mode AFM

The mode was developed as a method to achieve high resolution without frictional forces. The cantilever is oscillated near its resonance frequency as it is scanned over the sample surface. As the tip is brought closer to the sample at some point it will begin to intermittently contact ("tap") on the surface. This contact with the sample will cause the oscillation amplitude to be reduced. Once the tip is tapping on

the surface, the oscillation amplitude in general will scale in direct proportion to the average distance of the stylus to the sample. The oscillation level is set below the free air amplitude and a feedback system adjusts the cantilever-sample separation to keep this amplitude constant as the tip scans laterally across the surface. Because the contact with the sample is only intermittent, the stylus exerts negligible frictional forces on the sample.

Lateral Force Microscopy (LFM)

LFM is a modification of standard direct contact topographic imaging, in which the sideways forces on the probe are imaged. This shows changes in surface friction as well as enhanced contrast at edges. It is sometimes called "frictional force microscopy". LFM is used in conjunction with topographic imaging to show changes in material and helping to interpret images.

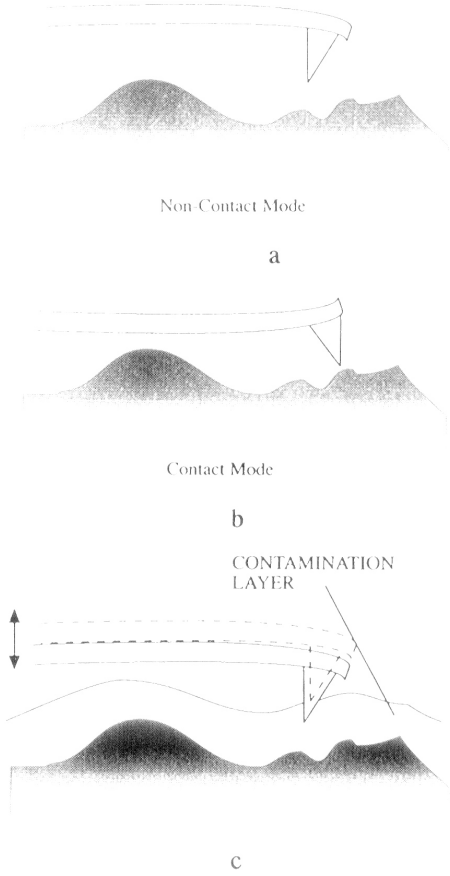

Figure 3: (a) In non contact mode, the AFM probe is pulled towards the surface primarily by capillary forces on contaminated samples and Van de Waals forces on clean samples. (b) In contact mode, the AFM probe is in the repulsive force region and the cantilever is pushed away from the sample. (c) The effect of surface contamination on the probe. The probe is pulled towards the surface by capillary attraction within the contamination layer.

Constant Force AFM

Images are obtained by maintaining a fixed force on the cantilever while the image is acquired. High responsiveness of the feedback electronics is required. Constant force mode is used to measure large areas and large surface features.

Variable Force AFM

Images are obtained when the probe end of the cantilever moves up and down during a scan, while the responsiveness of the piezoelectric ceramic is low. This mode is used for scanning small areas (100 Å x 100 Å).

THE PRESENCE OF ARTIFACTS

Artifacts in SPM images can be due to surface contamination, tip geometry and/or probe/sample adhesion.

- Sample surface contamination

The sample surface in ambient conditions will have a thin contamination layer. This layer is composed of water and other contaminants and its thickness ranges from 25 Å to 500 Å. As a probe tip is moved toward a sample surface that has a contamination layer, at some point it is pulled strongly toward the surface by capillary attraction within the contamination layer. Thus the attractive forces are much greater when a contamination layer is present than when the surface does not have this layer (Fig. 3c).

- Tip geometry

The shape of the probe tip is critical to AFM imaging, since the image is the result of both sample surface and probe shape. A very dull large radius, low aspect ratio tip will have a large area that interfaces with the contamination layer, resulting in very strong attractive forces. A sharp, high aspect ratio probe tip will have a much smaller area that interfaces with the contamination layer. Thus, sharp probes have a lower capillary attraction with the sample, since they have a small area of contact with the contamination layer (Fig. 4).

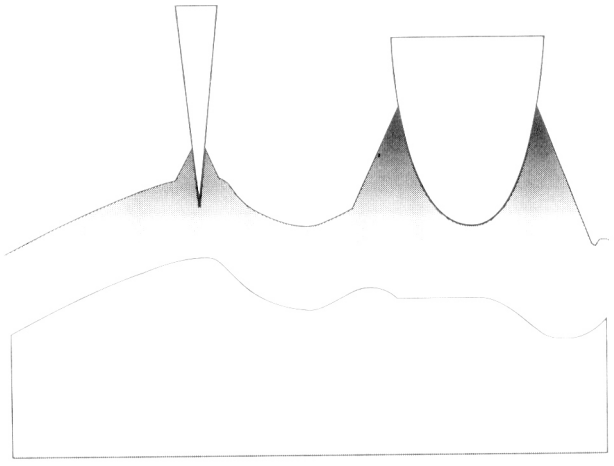

Figure 4: A sharp tip has a much smaller wetted surface than a broad tip, resulting in lower forces from capillary action.

- Nature of sample

The sample itself affects the probe/sample forces. Some samples are more likely to have surface contamination than others. In addition, some samples develop a static

electric charge readily and this can have a significant effect on the probe-sample interaction, making AFM imaging difficult.

APPLICATION OF SPM TO BIOLOGICAL SAMPLES

Since the first STM images of DNA were published in 1984, there have been many reports devoted to the STM and AFM imaging of a wide range of specimens (Edstrom et al., 1990). Molecular resolution images have been reported for a range of biomolecules including proteins, lipids, nucleic acids and biomedical polymers (Butt et al., 1990). Lower resolution images have also been obtained from cellular systems and polymeric films. Recent review on biochemical and structural applications of AFM describes the most important biochemical and structural results obtained during the last twelve months using the standard, contact mode of AFM imaging and discusses latest technical advances that are likely to play a major role in the study of biological systems in the near future (Bustamante et al., 1994).

One of the main aims of biological SPM is the visualisation of structural features and their changes and to relate these to biological activity. It is therefore critical that sample preparation procedures will induce minimal alteration of the specimen structure, maintain its functionality and allow reproducible SPM imaging to molecular resolution.

The magnitude of the specimen-support interaction is particularly important in SPM samples, since if adhesion force is to low the SPM tip may simply sweep the specimen over the substrate and hence no stable image will be obtained. Sample immobilisation methods used for SPM imaging include fixation of the specimen by a thin platinum-carbon coat and chemical linkage via activated substrates/molecules using gold-thiol bonds, biotin-streptavidin bonds and self-assembled monolayers. Materials used as substrates for immobilisation of biological specimens include graphite, gold and mica. Graphite is used primarily as a substrate as its inherent surface flatness allows for accurate scanning of materials layered upon it. Gold acts as a very good substrate on which to place a variety of biomolecular materials, as the biomolecules may easily maintain their original morphological features without being modified to fit over or around artifacts. Mica, a common material, acts as a superior substrate for biomolecular sample, where the user may statically control adhesion of biomolecules to the substrate surface.

THE USE OF AFM FOR STUDYING BIOFILMS AND BIOCORROSION

Atomic Force Microscopy has proved to be a very useful tool in elucidating the phenomena of corrosion due to the development of bacterial biofilms on metal surfaces. To date, two such studies have been reported in literature, one on copper (Bremmer et al., 1992) and another on stainless steel 316 (Steele et al., 1993; 1994).

The AFM observations of bacteria on copper by Bremmer et al. (1992) were carried out under liquid in the sealed fluid cell. It has been determined that bacterial strain

isolated from a corroded copper pipe colonised both polished and unpolished copper surfaces under batch culture conditions. Bacterial cells were shown to be associated with pits on the surface of the unpolished copper coupons.

In the work of Steele et al., (1993; 1994) a good level of correlation has been found between images of biofilms, formed by different species of bacteria on polished stainless steel coupons, obtained by AFM and by SEM. It has also been revealed that the three dimensional information of images from the AFM technique render it superior to the SEM, especially regarding its potential for quantitative assessment of any surface damage that occurred in the form of pitting (Steele et al., 1993). Recent studies demonstrated that AFM can be used to determine not only the depth and angle of pits formed on surfaces of stainless steel beneath biofilms grown in the presence of different bacterial consortia in different media, but the data collected during scanning can be processed to determine the effect of biofilms on surface roughness of the metal (Steele et al., 1994). In separate study, AFM investigation of biofilms developed on stainless steel by different isolates of marine sulphate-reducing bacteria (SRB) allowed direct visualisation of submicrometer features of bacterial cells (Steele et al., 1994). The AFM enabled imaging of the hydrated matrix of extracellular polymeric substances (EPS) to a degree that has not been previously demonstrated by any other microscopy technique (Steele et al., 1993; 1994). There is little doubt that this matrix was associated with bacterial cells, as it did not appear in any of the control samples.

AFM viewing of biological samples has been a subject of controversy over the production of artifacts. Recent research demonstrated that artifacts need not effect the imaging of bacterial biofilms, however interpretation of the images acquired has to be treated with discretion (Steele et al., 1994).

As stated earlier, studies of biofilms by using SEM techniques provide useful information regarding the distribution of cells within biofilms. However, the required preparation of a sample causes its considerable shrinkage and facilitates introduction of artifacts. Environmental scanning electron microscopy allows observation of fully hydrated, living biofilms. Although the biofilm matrix is preserved during ESEM viewing, the resolving power of this instrument is lower than that of the AFM. ESEM images of biofilms do not provide information regarding the morphology of the extracellular matrix, nor do they offer details of bacterial surface topography. Unlike other biological specimens, AFM studies of bacterial biofilms do not require sample pretreatment. There is no need to immobilise biofilms as the EPS facilitate bacterial attachment to the substratum. Due to the development of wet specimen cells, the observation of biofilms can be carried out under liquid, in their natural environment.
The following are examples of the application of AFM for investigating the formation of pure culture bacterial biofilms on metal surfaces.

Copper surfaces

Biofilms were generated on coupons (10 mm x 50 mm) in mannitol-glutamic acid media (mannitol 10 g, L-glutamic acid Na salt 2 g, monobasic KH_2PO_4 0.5 g, NaCl 0.2 g, $MgSO_4$ x7H_2O 0.2 g and 15 g of agar, $1l^{-1}$ H_2O; pH adjusted to 7.5 with 6N NaOH) in

batch cultures of a copper resistant strain of *Pseudomonas syringae* pNZ63 (culture supplied by Dr. C. Bender of the Department of Plant Pathology at Oklahoma State University in Stillwater, obtained from Dr. G.G. Geesey of the Centre for Biofilm Engineering at Montana State University in Bozeman). Copper surfaces were polished to achieve 1µ finish. After 3 weeks of incubations at 22°C coupons were removed from bacterial cultures and imaged in air using AFM (Topometrix) operating in contact mode, at a 75 µ scan range. Figure 5 shows micrographs of cells of *P. syringae* colonising surface of copper. The collected data is presented at different magnifications, either as a 2-dimensional scan (Figure 5a,b), or processed to obtain a 3-D image (Figure 5c). Bacterial flagella, and what appears to be fragments of amorphous matrix, can be clearly seen spreading on the surface.

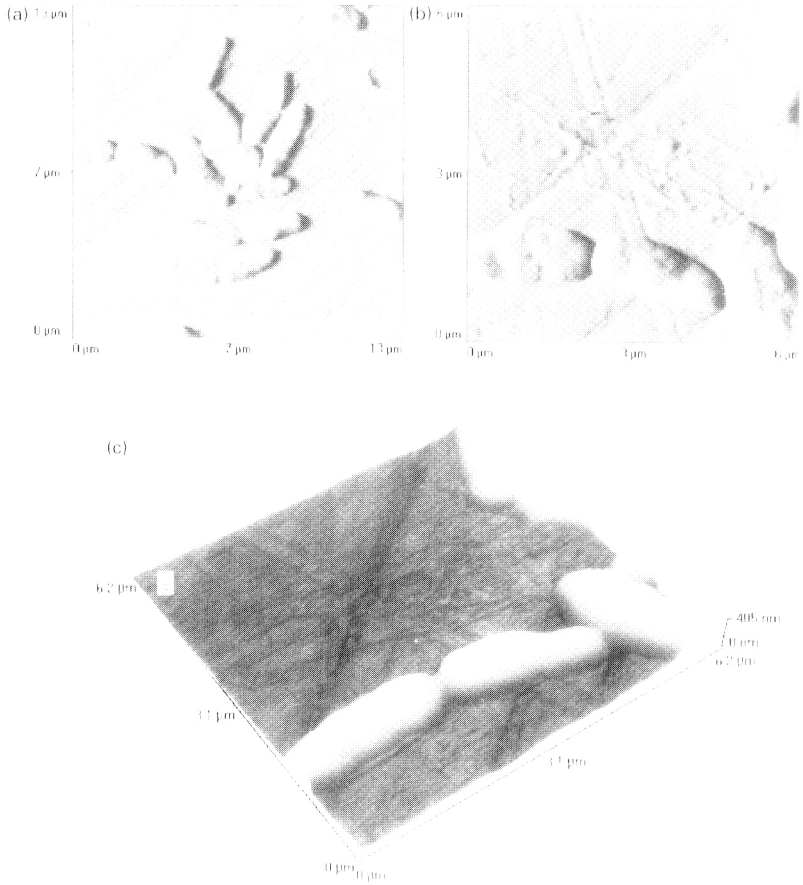

Figure 5: AFM micrograph of correr resistant *Pseudomonas syringae* cells forming biofilms on copper surfaces exposed to bacterial culture for one week. The auired data was processed to obtain different magnifications of two dimensional (A,B) or three domensional (c) images of bacterial cells. Flagella, as well as fragmented amourhous matrix, are clearly seen sperading on the surface.

Surfaces of stainless steel

Marine species of sulphate reducing bacteria (SRB), Ind1, isolated and purified as described elsewhere (Beech and Cheung, 1994) were grown in Postgate medium C (Postgate, 1994) at 30°C. Coupons made of stainless steel 304 (10 mm x 30 mm) were

placed in 5 day old SRB batch cultures and incubated for 21 days. Biofilms developed on surfaces of steel were viewed under AFM, as described for copper. Plates 2 and 3 demonstrate the capability of using AFM to collect information which enables not only qualitative but also quantitative analysis of the image such as measurements of the biofilm thickness (Figure 6), roughness of the substratum and depth and size of pitting (Figure 7).

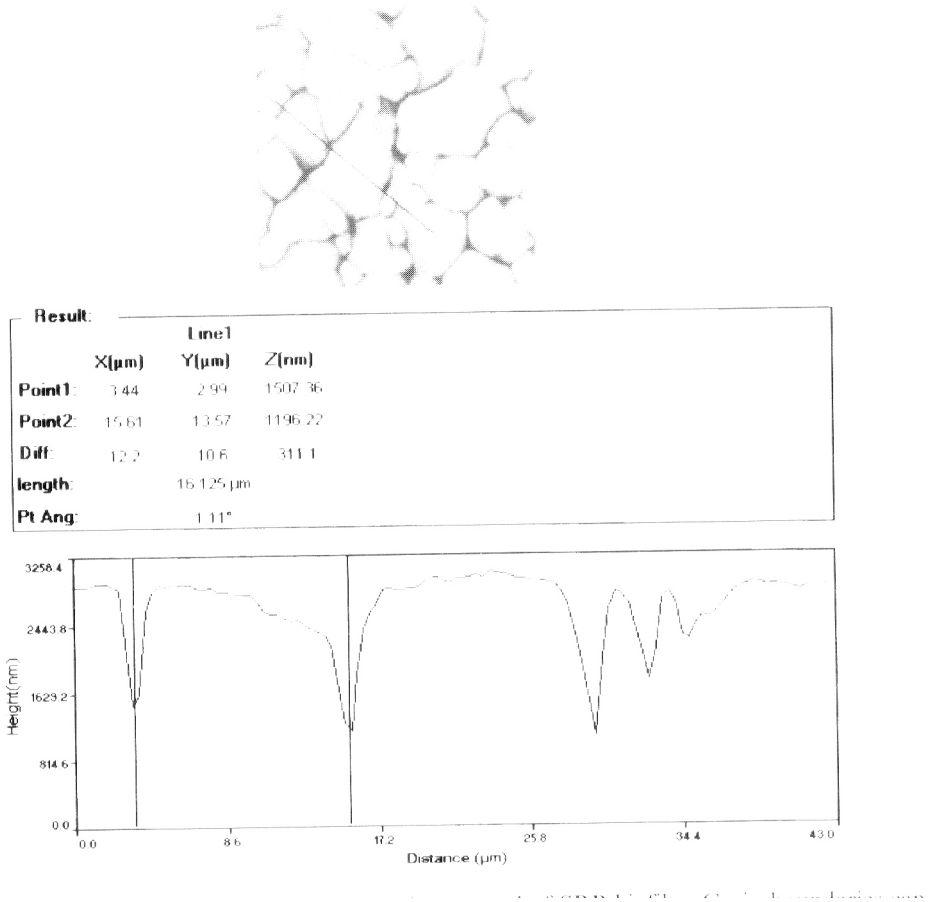

Figure 6: AFM image on the surface of stainless steel 304 after removal of SRB biofilm. Grain boundaries can be seen. The size of individual grains and the width and the depth of gaps between grains can be measured as shown by the profile obtained across the marker line.

There is little doubt that the continuous development and improvement of SPM techniques opens up great possibilities for investigation of microbe/material interactions. These type of studies will allow better understanding of the fundamental aspects of deterioration of metals due to the biofilm formation, thus helping in designing novel, more effective and environmentally acceptable biocorrosion control and prevention measures.

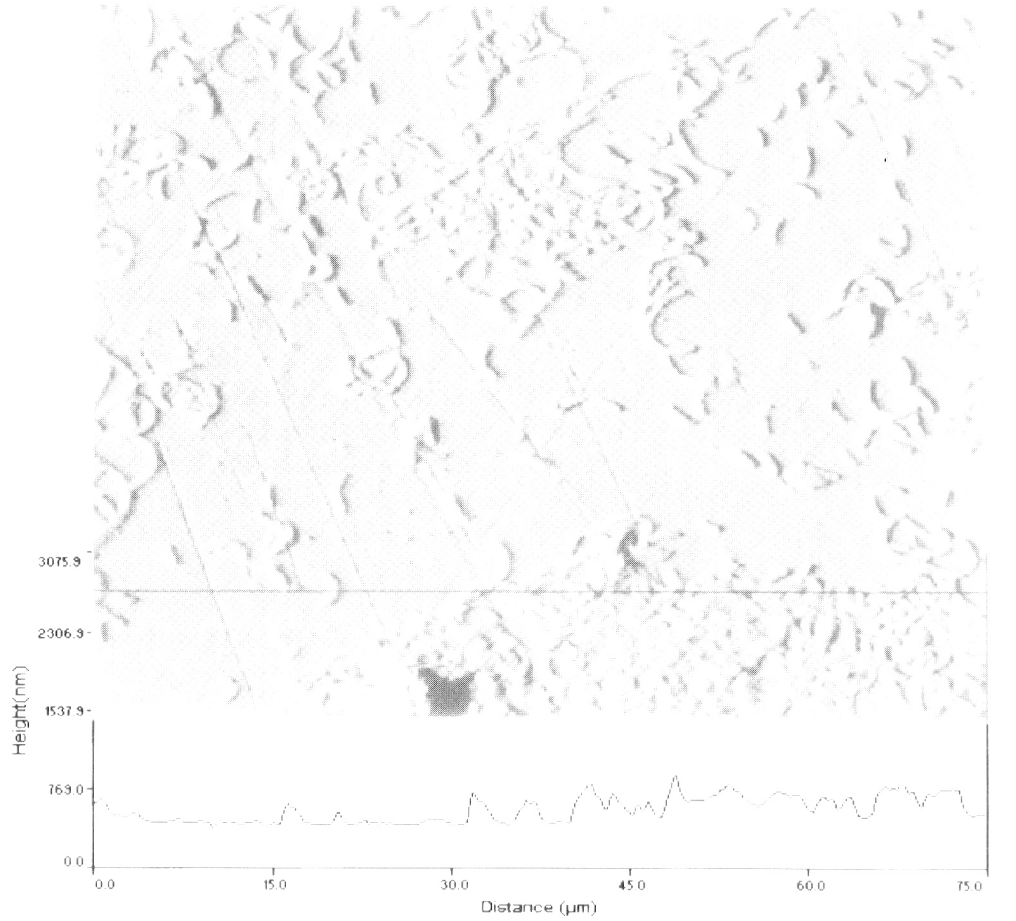

Figure 7: AFM contact mode image in air of a three week old biofilm formed on the surface of polished 304 stainless steel by a marine isolate of sulphate reducing bacterium. Profile of the surface roughness across the line is given, allowing the measurement of the size of indivdual bacterial cells and the thickness of the biofilm.

RECENT AFM RESEARCH

Biologists have used AFM in combination with light microscopy (transmitted bright field, epifluorescence and surface interference) to gain both topographical and visual information from the sample (Vesenka *et al.*, 1995; Nagao and Dvorak, 1998). However, the diffraction limit of optical microscopy does not allow the observation of fine features. The final SPM technique, scanning near optical microscopy (SNOM) uses a fibre optic probe which allows the optical diffraction limit to be breached (Lewis *et al.*, 1995; Lieberman *et al.*, 1996). When the topographical information is simultaneously collected with fluorescence imaging of the cells, the combination is known as SNFM (Scanning near-field fluorescence microscopy, Monobe *et al.*, 1998). The use of SNOM for the study of cell biology, which includes the investigation of *E. coli* containing green fluorescent protein (GFP) (Muramatsu *et al.*, 1996; Subramaniam *et al.*, 1997) and the use of the DNA dyes DAPI, Hoechst 33342 and ethidium bromide (Kirsch *et al.*, 1998) has recently been reviewed by Subramaniam *et al.* (1998). Ben-Ami *et al.* (1998) describe the first simultaneous imaging of bacteria

using SNOM and AFM, also known as SNOAM (Tamiya *et al*., 1997). Unstained bacteria were imaged and a comparison made with conventional AFM in both air and aqueous media. Certain internal features (endospores), and external ones (possibly the cell wall), not visible using AFM, were imaged using SNOM. Haydon *et al*. (1996) have described a confocal version of SNOM, terming it near-field confocal optical spectroscopy (NCOS).

AFM is often used in preference to methods such as scanning electron microscopy (SEM) as the technique has several major advantages. Since the sample need not be electrically conductive, no metallic coating of the specimen is required. Unlike with SEM, no dehydration of the sample is necessary and biofilms may be viewed in their hydrated state. This eliminates the shrinkage of biofilm associated with imaging using SEM, yielding a non-destructive technique. The resolution of AFM is higher than that of environmental SEM where hydrated images can also be obtained, and extracellular polymeric substances may not be imaged with clarity. The application of AFM for the study of biofilms on metal surfaces has been reviewed by Beech (1996).

When operated in contact mode, the original mode of operation (Binnig *et al*., 1986, 1987; Abraham *et al*., 1988), the AFM can be used to image samples on the atomic level (Bachelot *et al*., 1997). This mode of operation was improved upon by non-contact AFM (resonant or attractive mode) in 1987. Here a vibrating tip is oscillated at its resonant frequency to produce an image (Bachelot *et al*., 1997). The lateral resolution is generally limited to a few nm due to the larger distance (> 2 nm) between sample and tip, however the method allows imaging of more delicate samples. The third mode of operation is known as Tapping mode™ (trademark of Digital instruments) AFM or TMAFM, and is a compromise between contact and non-contact AFM (Zhong *et al*., 1993; Hansma *et al*., 1994; Constant *et al*., 1994). The cantilever is oscillated with larger amplitude than in non-contact mode (several tens of nm) and a larger cantilever spring constant is used. Periodically, due to these differences, the tip can cross the large range force field and contact the surface under study. The large tip motion makes the mode insensitive to shear forces and their destructive influence. Shear force AFM was developed for use in SNOM. Since the optical fibre probe is so delicate, it is impossible to use it with forces acting perpendicular to the surface. In shear mode, the probe vibrates parallel to the surface (Betzig *et al*., 1992; Toledo-Crow *et al*., 1992) with a vibration amplitude (typically a few nm) which is very sensitive to the tip-sample separation, so that a lateral resolution better than 10 nm may be obtained. A new mode of operation is now available, known as MAC mode. The Magnetic A/C (MAC) mode of AFM imaging, marketed by Molecular Imaging, was first reported by Zhong *et al.* (1993). This mode has a great advantage over TMAFM. In tapping mode, the oscillation of the tip results in mechanical excitations of the microscope as there is a damping effect due to the liquid present (Han *et al*., 1996), disturbing the position of the laser beam (Florin *et al*., 1993). This problem is overcome by directly driving the cantilever - a magnetic coating is applied and the cantilever deflected using a solenoid placed underneath the sample (Jarvis and Tokumoto, 1997). Using MAC mode there is no adhesion even to extremely sticky protein-coated surfaces, allowing imaging

previously unobtainable with AFM (Lindsay *et al.*, 1998). MAC mode has allowed researchers to develop the use of carbon nanotube tips which break when used in TMAFM due to the large acoustic vibration (Li *et al.*, 1998). Carbon nanotube tips have a well defined tip unlike those made of silicon nitride or silicon, and allow higher resolution imaging. As an added bonus, the tips are more resistant to crashing into the sample, decreasing the cost of imaging.

Adhesion Studies

Adhesion is a very important part in the process of biofilm development, hence, the object of intensive studies. Morra and Cassinelli (1996) used AFM to investigate the effect of the surface upon adhesion of the bacterium *Staphylococcus epidermis*, implicated in catheter-related urinary tract infections, finding that electron donor-electron acceptor interactions play a large part in the adhesion process. Gorman *et al*. (1997), studied the same bacterium and the influence of the conditioning film upon catheter material, measuring the surface roughness employing AFM. Hyde *et al.* (1997), also used AFM to correlate the effect of surface roughness and contact angle upon bacterial adherence and removal from fluorinated polymers, stainless steel, glass and polypropylene. Frank and Belfort (1997) measured the intermolecular forces between two layers of adsorbed EPS. This work allowed study of the effect of seawater on EPS, providing information on the structure of the conditioning layer upon which biofilms grow. Baty *et al*. (1997) reported the use of AFM to image mussel adhesive proteins and study the mode of adhesion to polymers. Comparison of the images obtained with AFM contact and tapping modes allowed observation of the effect of hydration upon such a layer. It was found that dehydration had a pronounced effect upon structure of the protein film on one polymer, but not on another. Bowen *et al.* (1998) report the first use of a single, living, immobilised cell as a "cell probe" for the study of cell-surface adhesion in the presence of a liquid environment. Using different cells will allow measurement of key parameters in the fundamental study of cell adhesion, including: the strength of cell-surface interactions, the time of development of adhesive contact, the influence of pH, ionic strength, effect of substratum (type, roughness, preparation, coatings), effect of cell life cycle and growth conditions, and finally the effect of weakening adhesion. The technique promises a new method of screening innovative antifouling materials and coatings. Tapping mode AFM was applied by McDonald *et al*. (1998) to visualise protein (fibronectin) binding to titanium implant surfaces, which is an important step in subsequent cell attachment. Recently, Steele *et al.* (1998) proved by a high-resolution AFM study of the surface of the Martian meteorite ALH84001 that the images of "biofilms" formed by alleged ultrananobacteria reported by team at NASA were not artefacts created by the SEM sputter coating process. Whether the features observed by McKay *et al.* (1996) are indeed biological, remains to be resolved.

Assessment of antimicrobial action.

AFM has been used by several researchers to investigate the effect and mode of action of antimicrobial agents on bacterial cells. Such studies include the action of penicillin on *Bacillus subtilis* (Kasas *et al.*, 1994), the effect of glutaraldehyde on

aerobic marine biofilms formed on stainless steels (Tapper *et al*., 1997), the influence of the antibiotic Cefodizime on *E. coli* (Braga and Ricci, 1998) and the action of Sterilox (superoxidised water) on *E. coli* and the sulfate-reducing bacterium *Desulfovibrio indonensis* (Tapper *et al*., 1998). Recently, Keresztes *et al.* (1998) studied the formation of metal sulfide layers on the surface of mild steel by the anaerobic sulfate-reducing bacterium *Desulfovibrio desulfuricans* with and without the presence of biocide.

Metal / microbe interactions.

Bremer *et al.* (1992) used AFM to demonstrate the presence of bacterial biofilms on polished and unpolished copper surfaces. Steele *et al*. (1994) and Beech *et al.* (1996) compared and studied the corrosion of stainless steel in the presence of different types of bacterial biofilms. Extracellular polymeric material was visualised as were micropits, with mixed bacterial cultures causing increased levels of corrosion as compared with pure ones. Maurice *et al.* (1996) described an AFM study of the bacterial interaction with hydrous Fe (III) oxides of the soil, which are known to control the movement of metals and organic pollutants through soils. The authors discussed the use of AFM in soil research and the problems encountered. Grantham *et al.* (1997) reported the use of AFM in investigating the microbially catalysed dissolution of iron and aluminium oxyhydroxide mineral surface coatings to gain a better understanding of bacterial subsurface mobility. Washizu and Masuda (1997) applied AFM to observe the interaction of iron-oxidising bacteria (IOB) and corroding metal, concluding that IOB tend to absorb to corrosion sites, and that they are activated by corrosion.

The Future of SPM.

Undoubtedly, the SPM methods have developed considerably in a relatively short time. A new form of SPM termed magnetic resonance force microscopy (MRFM) is currently under development. The MRFM could allow "non-destructive 3D imaging with Angstrom-scale resolution through the detection of single electronic or nuclear spins" (Noble, 1995). Such a device could be used for investigating proteins at a resolution better than that of traditional NMR, and could be used to investigate subsurface structures of cells. Reading *et al*. (1998) describe the potential for another new type of probe. Photothermal measurements using infra red (IR) radiation with a calorimetric analysis with scanning microscopy (CASM) instrument could provide IR microscopy well below the diffraction limit of IR, ultimately on the scale of 20-30 nm. The probe could also be used for point heating and thus pyrolysis of the sample, analysing the evolved gases using mass spectroscopy (MS) and even gas chromatography MS (GC-MS).
A final improvement to SPM is a change in the probe used in SNOM. Bergossi *et al*. (1997) describe a perturbative or apertureless SNOM probe made of tungsten, which allows a finer tip shape and hence a lateral resolution 10 times better than that obtained using an optic fibre.

ACKNOWLEDGEMENTS

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RELATION BETWEEN THE BIOCHEMICAL STRUCTURE OF MARINE BIOFILMS AND THE OXYGEN REDUCTION KINETICS ON STAINLESS STEELS

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ABSTRACT

It is now generally recognized that biofilm settlement on active-passive alloys immersed in natural seawater give rise to a depolarization of the oxygen reduction reaction on their surfaces.
This work summarizes the experimental results of several exposure tests executed directly in sea for the sake of singling out eventual relations between the changes in the oxygen reduction kinetics and the biochemical structure of biofilms.
The research activity, at first carried out in the Mediterranean Sea, had been later enlarged to other European seas during the Project BIOFILM entitled " Marine Biofilms on stainless steels: effects, monitoring and prevention", coordinated by CNR-ICMM and funded by the Commission in the MAST II Program.
The experimental results showed that the cathodic depolarization was everywhere in Europe linked to the amounts of Extracellular Polymeric Substances (EPS) in biofilms.
This observation, integrated with the results of other more recent field tests, has strengthened the hypothesis that the oxygen reduction depolarization, caused by the biofilm settlement on s.s. surfaces, could be the outcome of a catalysis from extracellular enzymes, probably oxydoreductases, intrapped and immobilized on the metal surfaces by the EPS matrix.

INTRODUCTION

It is generally recognized [(1)] that a marine biofilm, growing on stainless steels (s.s.) or similar active-passive alloys, causes in time a gradual depolarization of cathodic reactions.
The oxygen reduction, that at the immersion time of the material in seawater follows a kinetic of the "1st day" type, as the example in fig.1A, gradually change up to a kinetic of the "7th-10th day" type with the increase of exposure time and biological settlement.

This phenomenon causes on stainless steels in passive state an increase of free corrosion potentials up to +300/+400 mV (fig. 1B), that in turn, stimulates the onset of localized corrosion.
Figure 2 shows as an example, the increases in time of the free corrosion potentials registered on stainless steels in passive state during their exposition in different European seas (2).
As can be seen, in the first period of specimens immersion in the sea, the modalities of the rise in potentials can largely vary depending on the seawater flow rate, the temperature, the season, the site of the exposure tests, etc., but after 2-3 weeks, when the biofilms are everywhere completely developed , the potentials reached by the samples are identical.
This observation shows not only that the settlement of biofilms always causes a cathodic depolarization on stainless steels but that, everywhere, the phenomenon causes similar consequences in the corrosion behavior of materials.
Even if at the moment a general concordance on the corrosive effects of biofilm exists, the mechanisms through which the biofilm causes the cathodic depolarization are still unknown.

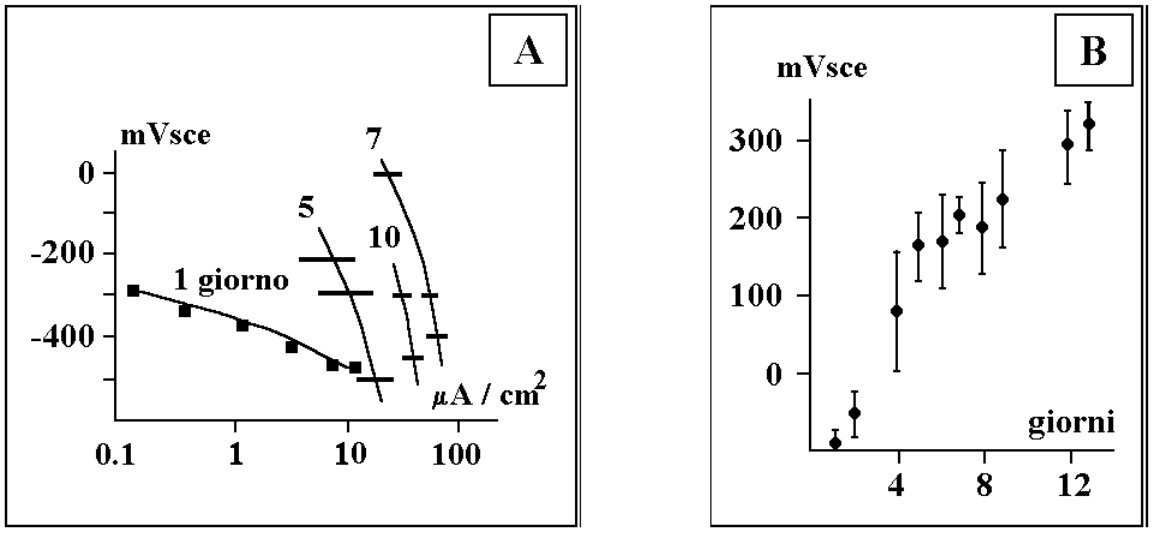

Fig.1 A) Evolution of the oxygen reduction kinetic on stainless steels in the course of their exposure in natural sea water.

B) Consequent evolution in time of free corrosion potentials of stainless steels in passive state. ($I_{passivity} = 10^{-8}$ A/ cm^2).

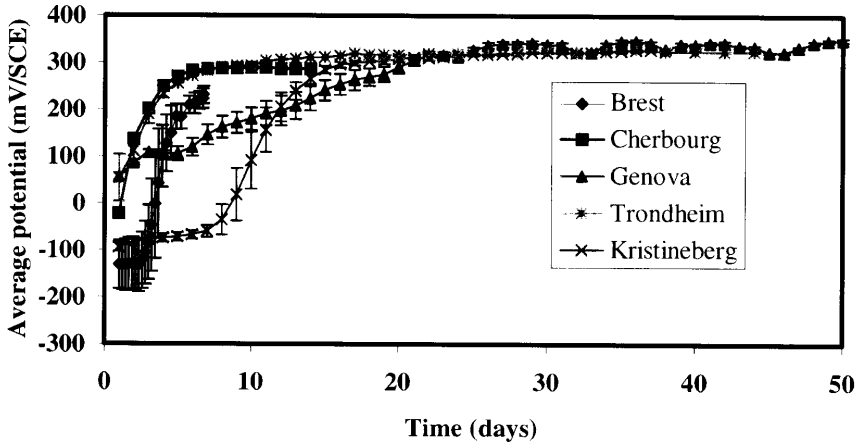

Fig.2 – Evolution in time of free corrosion potentials of highly corrosion resistant stainless steels (with at least 6% Mo) when_exposed in seawater in different European locations

Different hypotheses have been proposed in literature to explain this phenomenon and precisely:

- a depolarization of the oxygen reduction caused by enzymes [1,3] or ,more in general, by organic catalysts [4,5] present in the biofilm;
- a strong acidification of the medium, immediately near the steel surface, caused by the biological activity inside the biofilm [6];
- the production of high concentrations of hydrogen peroxide associated with low pH values, always of biological origin [7,8];
- the reduction of manganese oxides formed on the stainless steel surfaces following the activity of manganese oxidising bacteria[9,10].

Neverthless, field data which could confirm or deny the validity of the proposed hypotheses are still lacking. Therefore, to select the most realistic ones, it was decided to verify, through field tests carried out in the 5 European stations involved in the MAST program, whether some type of correlation between the evolution of the electrochemical effects and the biofilm "structure" exists.

With this in mind, the increase of potentials on stainless steels in passive state was taken as an indicator of the cathodic depolarization grade reached by the material, and the biofilm was detached from the samples only when pre-established potential values were reached.

The collected material was then analyzed using different analytical methods targeted to characterize the biofilm structure through measurements of settled microorganisms biomass, of their physiological activity, of the presence and relative incidence of specific populations, of the quantity of extracellular polymeric substances (EPS) characterized on the basis of their carbohydrate and protein content [11,12]...

The aim of this analytical approach was to identify the existence of some type of correlation between free corrosion potentials and the analytical data characterizing the biofilm structure, thus better focusing the biofilm fractions involved in the phenomenon.

The results of more recent field tests, which confirm the validity of the information obtained with the above described analytical approach, are finally described and discussed [13].

EXPERIMENTAL RESULTS

The analytical approach has permitted to establish that:

- the start up of the free corrosion potential ennoblement process definitely requires on the metal surface the presence of a bacterial settlement of the order of 10^7 – 10^8 cells/cm ^{2} ;
- the evolution of the electrochemical phenomenon is substantially indifferent to the biomass that the biofilm reaches on settlement;
- the physiological state of the biological settlement and the presence in it of algae do not work as determining factor for the onset of the electrochemical phenomenon on s.s.;
- the extracellular matrix alone of natural biofilms can be correlated in a significant way to the potential ennoblement. Figures 3 and 4 show, in fact, that the potentials of stainless steels in passive state increase with the protein and carbohydrate contents of the EPS matrixes independently of temperature, season, seawater flow rate, geographical position of the sampling site, type of stainless steel used,…

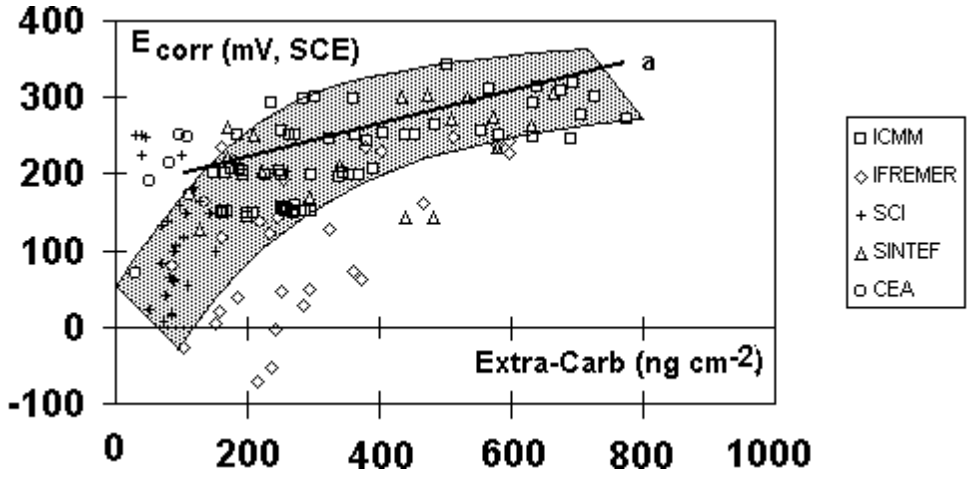

Fig.3 – Correlation between the free corrosion potentials and carbohydrate contents in the EPS matrixes of marine biofilms. The dotted line represents the correlationship previously established in Mediterranean Sea [14].

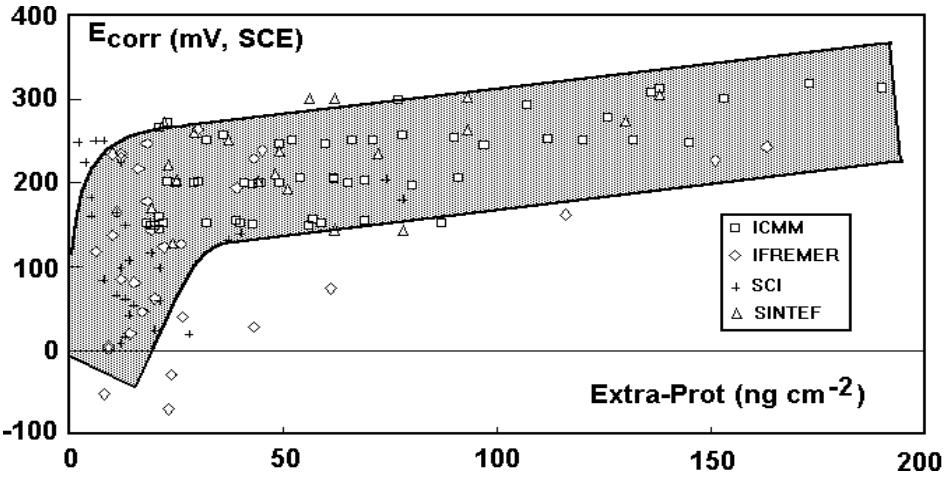

Fig.4 – Correlation between the free corrosion potentials and protein contents in the EPS matrixes of marine biofilms.

This observation, sprung from the analysis of biofilm samples taken in five different European seas, not only confirms what was already deduced from the data in Fig. 2, that is, the modification of the oxygen reduction kinetic induced by marine biofilms is a phenomenon of general validity, but also shows that everywhere, the phenomenon is correlated to some "bioproduct" held within the extracellular matrix of biofilms.

CONFIRMATION IN FAVOR OF A CATALYSIS OF THE OXYGEN REDUCTION INDUCED BY EXTRACELLULAR ENZYMES.

In a previous work [(2)] it was demonstrated that sodium azide added in sufficient quantities to seawater can eliminate the potential ennoblement induced by biofilm on stainless steels in passive state.

Sodium azide is a wide spectrum enzyme inhibitor, which blocks the respiratory chain of microorganisms with a consequent lethal action. Therefore, it was logical to ask if the disappearance of the electrochemical effect was due only to the death of microorganisms sticking to the metal surfaces, or instead to the inhibition of the extracellular enzymes, eventually trapped in the biofilm EPS matrix , able to catalyse the oxygen reduction.

To discriminate between the two possibilities, samples of highly corrosion resistant stainless steels were exposed in seawater and once the potential ennoblement was completed, the samples were treated first with a biocide as glutaraldehyde at 2.5% and then with sodium azide (0,01M).

Glutaraldehyde not only works as a potent biocide, but it causes a sort of "cross linking" on the EPS matrixes, such to immobilize eventual extracellular enzymes contained in it without losses of their activity.

The results of the test, shown in fig. 5, demonstrate that the treatment with glutaraldehyde, even though eliminating every form of life, left the electrochemical effect

of the biofilm unaltered which instead is cancelled by the successive biofilm treatment with sodium azide.
Considering that sodium azide is a reversible enzyme inhibitor, its inhibitive action should desappear when the product is removed from the solution.
This is exactly what happens when the biofilm is preliminarily treated with sodium azide and subsequently washed away with sterile seawater (fig.6).
The results illustrated in fig.5 and 6 substantially confirme the hypothesis of an enzyme catalysis of the oxygen reduction exerted by extracellular enzymes present in the EPS matrix where the electrochemical effects induced by the biofilm disappear with the treatment of sodium azide and reappear when the inhibitor is eliminated from the biofilm matrix with repeated washings in sterile seawater.

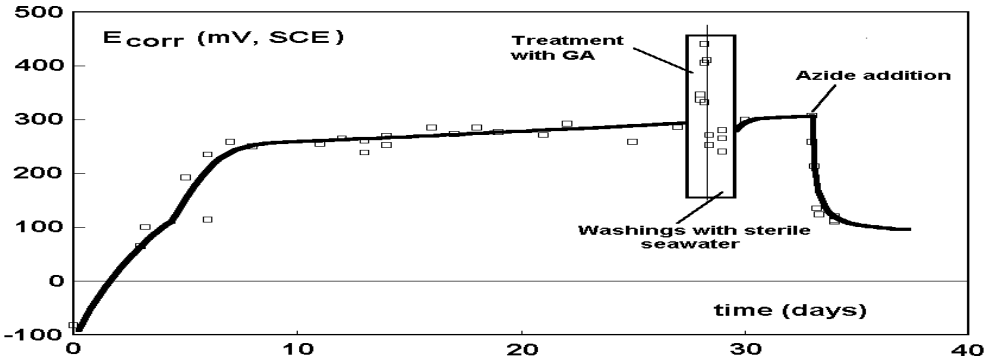

Fig.5 – Effect of an immobilization treatment of biofilms with glutaraldehyde and their subsequent treatment with sodium azide.

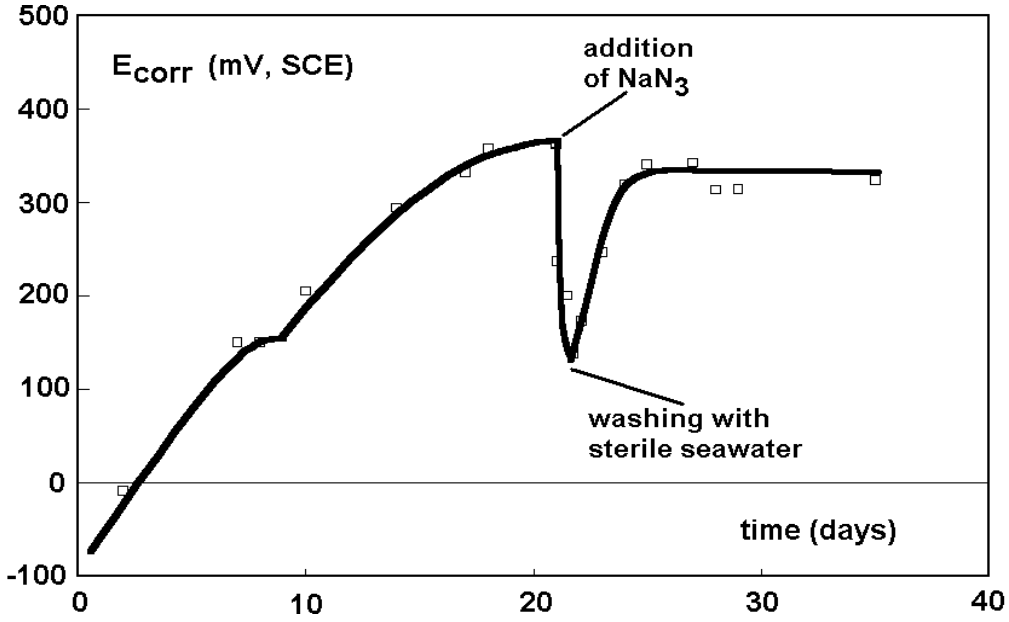

Fig.6 – Reversibility of the inhibition induced by sodium azide

ENZYMATIC SYSTEMS SUSPECTED TO INTERVENE IN OXYGEN REDUCTION.

It is well known that a complete reduction of oxygen into two water molecules, with the exchange of 4 electrons, does not have any negative effect on living cells, while a partial reduction with steps of 1 or 2 electrons, accompanied with the production of superoxide radical anions ($O_2^{\circ -}$) and hydrogen peroxide (Table 1), can intervene on cell membrane structure with consequent toxic effects.
It is evident that in this last case all aerobic organisms will be forced to defend themselves by producing suitable enzymes as superoxidismutases (SOD) which transform the radical superoxide in hydrogen peroxide and catalases and peroxidases which transform hydrogen peroxide into molecular water and oxygen according to the scheme shown in table 2.

Tab. 1

Complete oxygen reduction	$O_2 + 4\ H^+ + 4e^- \rightarrow 2\ H_2O$
Sequence of steps of partial oxygen reduction	$O_2 + e^- \rightarrow O_2^{\circ -}$ $O_2^{\circ -} + 2H^+ + e^- \rightarrow H_2O_2$ $H_2O_2 + H^+ + e^- \rightarrow H_2O + OH^\circ$ $OH^\circ + H^+ + e^- \rightarrow H_2O$

Tab. 2

Catalyser	Catalyzed reaction
Superoxidismutase (SOD)	$2\ O_2^{\circ -} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2\ H_2O_2 \rightarrow 2H_2O + O_2$
Peroxidase	$H_2O_2 + DH_2 \rightarrow 2\ H_2O + D$

A preliminary evaluation of the proteins held in the natural biofilm EPS matrix, carried out with electrophoresis on SDS-PAGE gels, put into evidence a discontinued distribution of bands between 15,000 and 100.000 daltons in an interval of molecular weights consistent with the units and sub-units of oxidoreductase enzymes above mentioned.

The SOD, catalase and peroxidase activities in the EPS matrixes of natural biofilms were finally evaluated with the usual spectrophotometric methods reported in SIGMA catalogues [15] and the decreasing order SOD> catalase > perossidase was established.
These preliminary data showed that only SOD activities point out some correlation with free corrosion potential ennoblement induced by biofilm adhesion (fig.7).

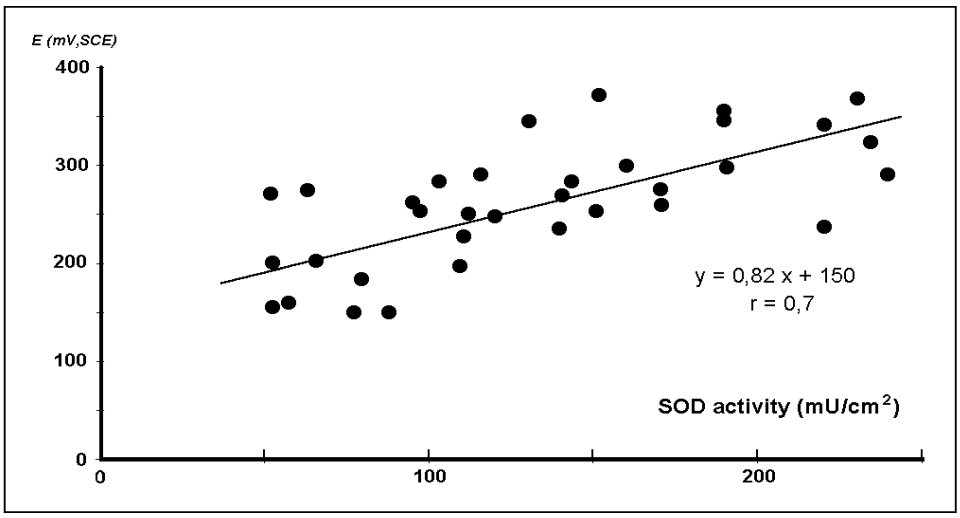

Fig.7 – Correlation between free corrosion potentials and SOD activity measured in biofilm EPS matrixes

These preliminary data, which necessitate further confirmation, seem to assign SOD a fundamental role in biocorrosion but " a priori" the possibility of a cascade-type enzymatic mechanism, which involves all the oxidoreduttases before introduced, cannot be excluded.
Future research will focus on bioeletrochemical studies directly applied on the EPS matrixes of natural biofilms, suitably concentrated and purified .

CONCLUSION

- The results of these field and laboratory tests suggest that oxygen reduction depolarization induced on stainless steels by the formation of natural marine biofilms is caused by extracellular enzymes trapped in extracellular polymeric substances (EPS) of biofilms;
- Spectrophotometrical analyses of the enzymes activities presumably involved in oxygen reduction, such as superoxidismutases (SOD), catalases e peroxidases, have demonstrated an effective presence of oxidoreduttases in natural biofilm EPS matrixes characterized by activities decreasing in order SOD> catalase>peroxidase.

- Some preliminary field data suggest that only SOD activity is correlated in a significant manner to the free corrosion potential ennoblement on stainless steels in passive state, seen as an indicator of the depolarization grade reached in oxygen reduction under biofilm action.

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Microbially Influenced Corrosion of Industrial Materials
European network

ELECTROCHEMICAL MICROSENSORS AND MICROELECTRODES : APPLICATIONS IN BIOFILM ANALYSIS

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The presentation is divided into two parts : the first part (A) is devoted to a brief overview of applications of microsensors and microelectrodes to the domain of biofilm analysis. The second part (B) deals with the state of the art of microelectrodes in the field of electrochemistry.

PART A : STATE OF THE ART IN BIOFILM APPLICATIONS

A distinction is made between microsensors and microelectrodes. Electrochemical microsensors are complex devices designed to be selective of only one component. They are generally organised into two groups according to the type of detection : potentiometric (potential difference is measured) or amperometric (current is measured). On the contrary, microelectrodes are electrodes, it means simple piece of material, of very small size and with no particular selectivity. Their selectivity depends on the electrochemical technique used.

I. ELECTROCHEMICAL MICROSENSORS [1]

I.1. Potentiometric detection

The potential difference is measured at the two extremities of an electrochemical chain containing an ion-selective element. For instance :

Reference electrode / Internal solution / Ion-selective element / External solution / Reference electrode

The internal solution contains the ion I, that must be detected, with a stable and well known activity ($a°_I$). The external solution is the solution to be assayed. The potential difference ΔE is linked to the logarithm of the activity of ion I (a_I) by the equation:

$$\Delta E = E_{background} + RT/z_IF \, Ln \, [a_I / a°_I]$$

or practically:

$$\Delta E = A + B \, Ln \, [a_I]$$

Any material whose potential difference between both sides specifically depends on the activities of ion I on both sides may be used as ion-selective element : glass (for pH electrodes e.g.), water-non-miscible solvent (ion exchanger or neutral carrier) stabilised on a porous support, crystal, non soluble salt, gel …

Some intrinsic disadvantages are linked to all types of potentiometric sensors :
- the response is proportional to the logarithm of the ion activity, and not directly to the ion activity,
- the response often depends on the nature and quantity of other ions present in solution,
- it is necessary to calibrate the sensor in the same conditions as used for the assay (not always possible).

Several microsensor have been proposed and some of them have been applied to measurement in biofilms or micro-organisms aggregates:
- micro-pH-sensors based on iridium oxide (tip size 20 µm) [2], or based on ion-exchanging liquid elements (tip size 1 µm) [3],
- microsensors selective to NO_3^-, NO_2^-, NH_4^+, Ca^{2+} have also been designed with different ion-exchanging liquid as sensitive element. Interference by other ions may drastically bias the measure in several cases [1,4].

The general characteristics of these microsensors are :
- Tip size around 1 µm
- Spatial resolution around 5 µm
- Cumbersome manufacturing procedure
- Life time: a few days

I.2 Amperometric detection

The potential of the working electrode is controlled with respect to a reference electrode by the electrochemical set-up, and the current is measured. When operating conditions are well chosen, the current is directly proportional to the concentration of the species to be detected.

I.2.1. O_2 and H_2S microsensors

Gold electrode deposited on platinum and coated by an O_2-permeable membrane has been used as oxygen sensor. The basic reaction is the reduction of oxygen into water at 0.8 V *vs*. Ag/AgCl :

$$O_2 + 4\ H^+ + 4\ e^- \rightarrow 2\ H_2O$$

Mounted on a micro-manipulator, a 10 µm sized sensor was used to determine oxygen profiles in a 300-µm thick biofilm [5].
An amperometric sensor has also been proposed to measure H_2S concentrations, via a reaction with hexacyanoferrate.

I.2.2. Microbiosensors

Microbiosensors are amperometric sensors involving a biochemical reaction. Glucose microsensors have already been applied to analyse biofilms and micro-organism aggregates [6-8].

II. MICROELECTRODES

II.1. General principle

In the framework of biofilm analysis very classic assumptions are generally used :

- potential is chosen in order to achieve a very fast electrochemical reaction on the electrode surface, and concentration of the electroactive species is assumed to be nil on the electrode surface,
- under these conditions current is directly proportional to the mass flux of the electroactive species,
- current is proportional to the surface area of the electrode.

Strictly speaking, this last assumption is only valid for classic macro-electrodes and it cannot be used for microelectrodes. This is discussed in part B of the presentation.

II.2. Mass transfer rates and flow velocities

II.2.1. Results

A 3-10 µm size platinum microelectrode has been used to measure mass transfer rates of hexacyanoferrate inside a biofilm. Plotting the map of local mass transfer rates supported a new description of biofilm structure, that should be composed of microbial cell clusters separated by interstitial voids. Tortuous water channels between the microbial clusters allowed convective flow and eddies to occur inside the biofilm [9]. A recent theoretical work allowed the map of local velocities to be derived from the map of mass transfer rates [10].

Recently, a 25 µm gold based mercury electrode has been used with a square wave voltammetric technique. A 10-µm depth resolution was claimed. Thanks to the efficient electrochemical technique, O_2, H_2O_2, dissolved manganese (hydro)oxides, Fe(II), Fe(III) and sulfides can be measured selectively with a detection limit of 20 µM [11].

II.2.2. Discussion

Measuring the oxygen concentration profile in a diffusion layer with a microelectrode has been tested by K.Rasmussen et al. [12]. No biofilm was involved in the experimental set-up, the oxygen diffusion layer was only created by reduction of oxygen on the surface of a graphite electrode. Important differences (around 80%) were observed between the values given by the microelectrode and the real mass flux values. Presence of the microelectrode in the diffusion layer was suggested to effect mass transfers. Assumptions used to derive mass flux values from the local measurements may also introduce some errors.

In a general way, the measure obtained from microsensors or microelectrodes must be interpreted with great caution because a lot of sources of error may exist :
- To measure small currents requires drastic experimental cautions: sensitive electrometer, shielding from electromagnetic interference, Faraday cage …
- Introduction of the tip into the film may create a dent in the biofilm, allowing bulk liquid to penetrate into the biofilm and to change the local concentrations,
- Calibration under well stirred conditions does not correspond to the real conditions of the measure,
- Water is not stagnant in the biofilm as it is generally assumed.

Moreover, even if they are generally used in the field of biofilm analysis, equations for classic (macro) surface area electrodes are not valid for microelectrodes. This is a very important point which is discussed in part B.

PART B : STATE OF THE ART IN ELECTROCHEMISTRY

When the dimensions of an electrode are decreased from millimetre to micrometer scale, many changes occur in the electrochemical behaviour.

I. INTRODUCTION

I-1 History and appellation

Although the advantageous properties of very small electrodes were recognised for many years, research in this area did not become very active until the late 1970s. Fleischman and co-workers of the University of Southampton [13] initiated much of this activity with an interest in understanding electrode mechanisms under conditions of high current density. Twenty years ago, few people recognised the advantages of using very small electrodes. Now, the literature contains many papers per years which increase our understanding of the properties of microelectrodes or demonstrate new types of application.

A microelectrode is an electrode with at least one dimension small enough that its properties, for example mass transfer regime, are a function of size. The concept of ultramicroelectrode (UME) is now widely accepted in the literature, but the limit between a microelectrode and an UME is badly defined. If the critical dimension of the electrode is the millimetre, it is called microelectrode. If the critical dimension of the electrode is rather the micron, one will call it ultramicroelectrode.

I.2 Commons types of ultramicroelectrodes

According to the geometry of the microelectrode, the diffusion field is different, contrary to a normal size electrode. Indeed, two types of diffusion exist : linear diffusion and spherical diffusion (figure 1).

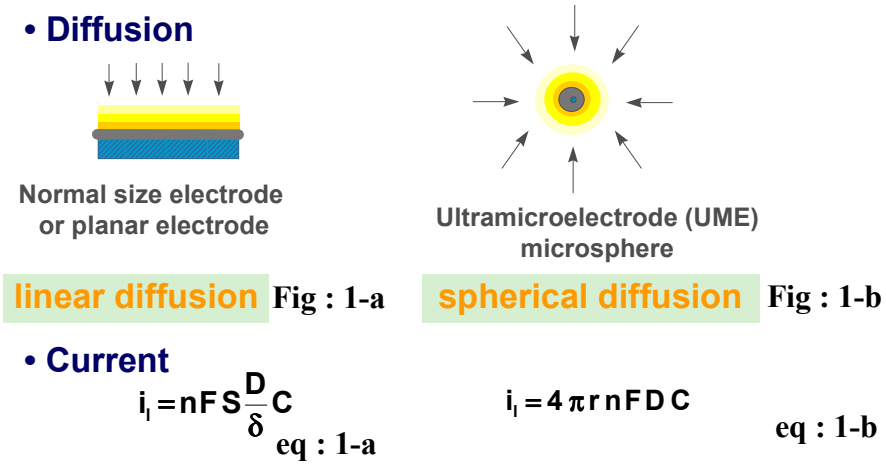


Figure 1 : Different types of diffusion field and comparison of the equation of the current between a planar electrode and a microelectrode

In the case of a planar electrode, the diffusion field is represented by figure 1-a. The diffusion is linear. In the case of a microsphere, each point on the surface is entirely equivalent, and hence the rate of diffusion is not a function of the position (figure 1-b). In the case of a planar

electrode, the current is a function of surface area (equation 1-a), whereas for a microsphere, the current is a function of the radius (equation 1-b). The more significant difference between a planar electrode and a microelectrode, is that the current given by a planar electrode is a function of the surface area, whereas the current given by a microelectrode is a function of the critical dimension. The common geometry of microelectrodes are microsphere, microdisk and microband (table 1).

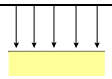
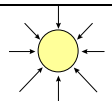
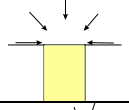
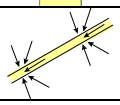
Type of electrode	Critical dimension	Geometry of diffusion field
Normal size electrode 	–	linear
Microsphere 	radius	spherical
Microdisk 	radius	spherical
Microband 	width	cylindrical

Table 1 : Common geometry of microelectrodes

The critical dimension are the radius for the microphere and the microdisk, and the width for the microband. In practice, it is very difficult to manufacture sphere and the theory of the microband is not easy because the geometry of the diffusion field is cylindrical. Therefore, microdisk is the most common microelectrode geometry.

In the case of a microdisk, the flux of material reacting is not uniform on the whole electrode surface. The flux of the outer circumference, so called spherical diffusion, is more significant than the flux to the central portion of the disk, so called linear diffusion. The diffusion is then convergent, but in many cases, it can be regarded as spherical.

II THEORY OF ULTRAMICROELECTRODES

II.1 Diffusion at ultramicroelectrode

II.1.1. Linear diffusion

In the case of linear diffusion, let us consider a planar electrode in an unstirred solution containing a base electrolyte and only one electroactive species. The linear diffusion may be described by Fick's second law. The solution may be found by Laplace transform techniques and it may be shown that the current is given by Cottrel's equation :

$$i(t) = \frac{n F S D_{ox}^{1/2} C_{ox}}{\pi^{1/2} t^{1/2}}$$

n is the number of electrons per molecule, F the Faraday constant, S the electrode area, D the diffusion coefficient of the electroactive species being electrolysed, C the concentration and t the time.

The current is inversely proportional to a time-dependent term.

II.1.2. Spherical diffusion

The electrode is a microsphere of radius r. In this experiment, diffusion of electroactive species to any spherical electrode may be described by Fick's second law in spherical coordinates. The current is given by equation :

$$i(t) = n\,F\,S\,D\,C\left[\frac{1}{(\pi D t)^{1/2}} + \frac{1}{r}\right] \qquad i_{(sphere)} = i_{(plane)} + \frac{n\,F\,S\,D\,C}{r}$$

In this case, the response is the sum of a steady state and a transient component, both of which being significant. At short time, the first term is larger than the second term. The current is inversely proportional to a time-dependent term. The UME behaves like an planar electrode : diffusion is linear and the current is given by Cottrell's equation. At long time, when t becomes rather large, the transient current decreases to a negligible value and the current reaches a steady state value given by the second term of the equation. The steady state current has a constant value which is inversely proportional to the radius of the electrode.

Between the two limiting time ranges, the diffusion regime is complex and the full equation must be used. Normally, it is advisable to avoid experiments in this time-scale. The complete equation then includes a time-dependent term, which corresponds to the linear diffusion, and a second time-independent term. This second term converges to a constant value which depends on the radius. Whereas the limiting current of a planar electrode is proportional to the surface, this equation shows well that steady state current for a microelectrode is a function of the radius.

II.2 Advantages of ultramicroelectrode

The small size of UME involves three main consequences : double layer capacitance is reduced, ohmic drop is minimised, and current density is very high. The third feature is induced by edge-effects, which correspond to the superposition of the linear and spherical diffusion types. The increase of the electroactive species flux on the electrode surface results in a better sensitivity of measurements. The first and second consequences are discussed below.

II.2.1. Effects of reduced capacitance

The charging current, which is due to the double layer capacitance of the electrode/solution interface, may be a major interference, since it may exceed the value of the faradaic current (the only part of the current relevant for the measure). The low capacitance of UME improves the ratio of the faradaic current to the charging current. This ratio increases when the radius of electrode decreases, or when time increases. However, the reduction of double layer capacitance makes it possible to use very high potential scan speed in voltammetric methods. This makes it possible to investigate rapid electron transfer and fast coupled chemical reaction. The microelectrode can consequently be used as sensor and to perform continuous measurements in environmental analysis or in vivo.

II.2.2. Effects of solution resistance

Solution resistance can severely distort electrochemical data and, until the advent of UME, precluded measurements in many organic solvents. When classic current values (I) are measured with macro-electrodes, using media with high resistance (R) results in high ohmic drops (I x R), which distort the measurement. With UME current values are very low and the

ohmic drop is consequently drastically reduced. No significant distortion is observed on the measurements, even in low conductive media, such as drinking water or organic solvents e.g..

II.3 Disadvantages of ultramicroelectrode

Microelectrodes present also some disadvantages. Indeed, the size of the electrodes being very small, it is very difficult to reproduce electrodes of identical size and geometry. Moreover, like the measured currents are very weak, it is necessary to amplify them and to work with very specific instruments (home-made amplifier, Faraday cage …).

III CONSTRUCTION AND USE OF ULTRAMICROELECTRODES

Only few microelectrodes are commercially available, and manufacturing them may present important practical problems.

III.1 Construction of microelectrodes

The intended use of microelectrodes determines the geometry, the size and the most desirable material. According to the studied medium, insulator has a very important role. In aqueous medium, one can indifferently uses glass or epoxy as insulator. In organic medium, only glass is used. The manufacturing technique, at the laboratory, varies according to the selected insulator [13-15]. On the industrial scale, UME are mainly manufactured by lithography pattern and electrodeposition [13,14].

III.2 Applications

Two examples of applications are presented here.

III.2.1. Microelectrodes in analysis

The first example deals with amperometric titration of hypochlorous acid with a microelectrode in an aqueous medium of low conductivity. The sensitivity obtained with a microdisk of radius 70 μm is 2 nA per ppm [15].

III.2.2. Microelectrodes in biological systems

The studies of Wightman and Amatore et al. represent nice examples of applications of UME in biochemistry. Wightman studied the detection of dopamine and oxygen in the rat brain [14]. Amatore monitored an oxidative response in a single living cell : he observed the variations in hydrogen peroxide and oxygen concentrations near the cell surface, when the cell was stimulated by puncturing its membrane [16].

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