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Kinetics of conditioning layer formation on stainless steel immersed in sea water

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Abstract: Adhesion of micro-organisms to surfaces in marine environments leads to biofouling. The deleterious effects of biofilm growth in the marine environment are numerous and include : energy losses due to increased fluid frictional resistance or to increased heat transfer resistance, risk of corrosion induced by micro-organisms, loss of optical properties, quality control and safety problems. Antifouling agents are generally used to protect surfaces from being affected by such a biofilm. These agents are toxic and can be persistent, causing harmful environmental and ecological effects. Moreover, the use of biocides and regular cleaning considerably increase the maintenance costs of marine industries.
An improved knowledge of the biofilm adhesion mechanisms is needed for the development of an alternative approach to the currently-used antifouling agents. The aim of this study is to characterise the chemical composition of the molecules first interacting with stainless steel during the period immediately following immersion in natural seawater and to elucidate the kinetics of the adsorbtion process. Proteins are shown to adhere very rapidly, closely followed by carbohydrates. The distribution on the surface of the organic molecules is also examined. The adsorbate on the surface is not so much a continuous film as a heterogeneous deposit, whose average thickness varies widely. The cleaning procedures used affect the adsorption kinetics. In particular, cleaning with hexane results in slower adsorption of nitrogen-containing species than does cleaning in acetone.

Keywords: Conditioning layer, seawater, protein, carbohydrates, surface characterisation, biofilm

Introduction

A material immersed in an aquatic environment is rapidly covered with a biological slime or biofilm. The first step, occurring within minutes to a few hours of immersion, corresponds to the spontaneous adsorption of organic and/or inorganic macro-molecules already present in the environment or produced by micro-organisms. This film is called the primary or conditioning film (Baier, 1972; Loeb and Neihof,1975; Taylor *et al*.,1997). However, the exact chemical nature, the kinetics of adsorption and the functional groups of the molecules involved in binding to the surface are not known. Using Multiple Attenuated Internal Reflection Infrared spectra of biomass on germanium exposed for ten minutes in seawater from Biscayne Bay (Florida, USA), Baier (1972) was the first to indicate that this conditioning film is essentially made of proteins. Fluorescence measurements carried out by Loeb and Neihof (1975) indicated that humic acids may also be present. More recently, on the basis of infrared spectral information, Taylor *et al* (1997) proposed the presence of large variety of adsorbed molecules (proteins, lipids, humic acids, nucleic acids, polysaccharides, aromatic amino-acids...) after 3 days of immersion in oligotrophic waters. However, they still considered it reasonable to model the primary film by an adsorbed protein layer. According to them, the composition of this conditioning film is highly dependent on the substratum nature, even after 3 days of immersion. Conversely, Little *et al*. (1985) found that the chemical composition and the quantity of adsorbed organic material are strongly dependent on the substratum nature during the first hour of immersion, but are completely independent of it after four hours (Maki, 1990However, the loosely attached material was not rinsed off and this study is consequently not concerned specifically with the adsorbed organic matter. Thus, the chemical nature of this adsorbed layer is still poorly characterised.
Information is also lacking concerning the exact role of this film on subsequent bacterial attachment. These adsorbed macro-molecules undoubtedly modify the substratum surface properties and a few papers emphasise the importance of surface charge modifications, wettability and surface free energy on the initiation of microbial colonisation (Dexter, 1979, Baier, 1980; Fletcher and Marshall 1982, Schneider (1997)).

A better knowledge of the chemical composition and of the kinetics of conditioning layer formation is a central requirement for a sound scientific understanding of biofilm growth and adhesion mechanisms. This would surely help in the development of an alternative approach to the currently used antifouling agents, which are generally harmful to the environment. Due to its complexity and to the interdisciplinary nature of the subject, this research requires a collaboration between the fields of material science, surface analysis, chemistry, marine microbiology and biochemistry. In this paper, we attempt to characterise the chemical nature and the distribution of the molecules first interacting with the surface. Further work, such as investigation of the adsorption of protein fragments, would still be needed to characterise fully the type of chemical interactions occurring. This study is concerned with the very first steps of biofilm formation on AISI 316L stainless steel immersed for short periods in natural sea water (2, 5, 8 and 24h). Analysis is performed using a broad range of surface characterisation techniques : X-ray Photoelectron Spectroscopy (AR-XPS) for chemical characterisation of the passivated metal surface and of the adsorbed organic species, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) for chemical characterisation of the adsorbed organic species, Infrared Reflection Absorption Spectroscopy (IRAS) for the description of the molecular bonds involved in the adsorbed layer present on the surface and of their possible change of orientation during film growth as described by Berrada (1990), Atomic Force Microscopy (AFM) to study the morphology, thickness and homogeneity of the organic film, and finally liquid drop contact-angle measurements to

investigate the hydrophilic/hydrophobic and acido-basic character of the uppermost layer of material. Particular emphasis is also placed on the importance of the surface preparation of the tested materials before immersion in natural sea water and of the unavoidable presence of a contaminant layer (the presence of such a layer is often ignored in scientific papers on similar topics).

Materials and methods

Surface preparation, immersion, transport

Square samples (1 cm x 1 cm or 5 mm x 5 mm) taken from a AISI 316L stainless steel plate (Goodfellow) were abraded with SiC emery paper (final stage 1200 grit) and diamond paste down to 1 µm, cleaned between each grade in a water ultrasonic bath, rinsed with acetone and ethanol, washed in three successive demineralised water baths at 50 °C under a sterile atmosphere near a Bunsen burner, and finally air-dried. Complementary tests with hexane rinsing in place of ethanol have also been performed. Hexane, 95%-ethanol and acetone were purchased from Fisher (analytical reagent grade), from Carlo Erba (reagent grade) and from Merck (analytical reagent grade) respectively.
The samples were immersed immediately after the surface preparation for short periods of exposure (2, 5, 8 and 24h) at different times of year in a bath that was continuously renewed with natural sea water. As the organic matter content of sea water varies a lot during the year, it was important to conduct these experiments in different seasons. However, the results included here essentially concern immersions in the summer (July). A complete analysis of the conditioning film formation as a function of seasons will be given in a forthcoming paper. The exposure periods, rinsing procedure and seawater temperature are summarised in Table 1. The samples lay flat on plastic netting in a 45 litre bath at Ifremer. Sea water was directly pumped from the Brest Bay and roughly filtered before being used to supply the experimental set-up in the laboratory. The renewal rate of sea water was around 1 litre/min. After exposition, the samples were re-immersed, either in the same solutions or in sterile sea water, in 40 ml containers and frozen to -20°C (in about 1 hour), in order to stop any further chemical processes at the metal-liquid interface. The term "0 hour immersion" used hereafter actually corresponds to an immediate freezing of the sample in natural sea water. The samples were then sent to the various laboratories by express delivery under dry ice (transfer time around 1 day). Before any analysis, the samples were rinsed in two successive sterilised and demineralised water baths in order to eliminate all loosely bound species, dried under clean air and immediately analysed. An exception is the AFM studies, which were performed under water (the transportation water was used).

Analytical Techniques

X-ray Photoelectron Spectroscopy (XPS)

The XPS analyses were performed with a VG ESCALAB Mark II spectrometer, using an Mg Kα X-ray source (1253.6 eV) and 20 eV pass energy. The reference energies were the $Au4f_{7/2}$ signal at 83.9 eV and the $Cu2p_{3/2}$ signal at 932.8 eV. A survey spectrum was first recorded to identify all elements present at the surface, then high resolution spectra of the following regions were recorded: Fe2p, Cr2p, Ni2p, Mo3d, O1s, C1s, Cl2p, Na1s, S2p and N1s. The

spectra obtained were fitted with reference spectra following the Shirley procedure (De Vito and Marcus, 1992). The C1s peaks were analysed according to the references given (Beamson and Briggs, 1992): C-C and C-H (285.0 eV), C-O and C=O (287.0 eV) and O-C=O (289.4 eV). The following values for the atomic sensitivity factors and attenuation lengths were used: Y_{Cr}/Y_{Fe}=0.70 (calculated using the XPS intensities of Fe and Cr emitted by the clean surface of the alloy under study), Y_{Ni}/Y_{Fe}=1.65, and λ_{Fe}^{met} = 10 Å, λ_{Fe}^{ox} = 17 Å, λ_{Cr}^{met} = 12 Å, λ_{Cr}^{ox} = 19 Å, λ_{Ni}^{met} = 9 Å, λ_{Ni}^{ox} = 15 Å. All attenuation lengths were estimated after Seah and Dench, (1979). Calculations of the type developed by De Vito and Marcus (1992) yield the compositions and thicknesses of the oxide layers. The analysed surface was a spot of size 2000 µm x 6000 µm and XPS analyses were performed twice for each type of sample.

Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Positive and negative ToF-SIMS measurements were performed with a TFS-4000MMI (TRIFT) spectrometer from Phi Evans (Bertrand and Weng, 1996). For these measurements, the sample was bombarded with pulsed primary $^{69}Ga^{+}$ ions (15 keV). The spot diameter was about 2 µm. The secondary ions were accelerated to ± 3 keV by applying a bias to the sample. The spreading of the initial energies of the secondary ions was compensated by deflection in three electrostatic analyzers. To increase the detection efficiency for high-mass ions, a post-acceleration of 10 keV was applied at the entrance of the detector. The analysed area was a 130 µm x 130 µm square . The information depth in ToF-SIMS has been shown by Delcorte A *et al*. (1996) to be inferior to 5 Å for molecular species and up to 20 Å for ionic species. Four random areas were analysed on each sample. With a data acquisition time of 5 min, the total ion dose was about 10^{12} ions/cm^2, which ensures static conditions (Briggs and Hearn, 1986). With the present samples and analytical conditions, the mass resolution was about 5000 m/Δm at m/z = 29. This highly facilitates the assignment of ions having the same nominal mass but different compositions, e.g. hydrocarbons, oxygenated nitrogen-containing fragments, etc. The relative intensities of specific positive ions were estimated using Equation 1 where $Itotal$ and $\sum Icontam$ are respectively the sum of the intensities of all secondary ions and the sum of the intensities of all specific peaks of polydimethylsiloxane: PDMS, $(Si(CH_3)_2\text{-}O)_n$, while I_X is the intensity of the peak x.

$$Irel = \frac{I_X}{Itotal - \sum Icontam} \qquad [1]$$

Fourier transformed infrared spectroscopy (FTIR)

FTIR measurements were carried out in the reflection mode at grazing incidence (6°) using a Fourier transformed infrared spectrometer (NICOLET, Magna 550) with an MCT wide band detector. A typical spectrum was obtained by averaging the signal over 600 scans in 6 min. at a resolution of 4 cm^{-1}. All spectra were divided by a background spectrum recorded on a sample which had been immersed for a few seconds in distilled water and dried.

Surface-energetic characteristic determination

The “drop” method, using a Krüss G40 goniometer, was applied to measure the contact angles of pure liquids of known surface energies (water, formamide, di-iodomethane, α-

bromonaphthalene) on the stainless steel samples, before and after immersion. The liquid/vapour surface tension parameters of the liquids are given in Table 2. The data come from (Van Oss *et al.*, 1988) except the value γ^{LW} of α-Bromonaphthalene liquid which was measured at INRA (Khayat *et al*., 1997) using a Krüss processor tensiometer K12, Germany, and the Wilhelmy plate method (Wilhelmy , 1863). The surface-energetic characteristics of the samples are yielded by the Young-van Oss equation (Van Oss *et al.*, 1988):

$$\gamma (\cos \theta + 1) = 2 \{ (\gamma_S^{LW} \gamma_L^{LW})^{1/2} + (\gamma_S^- \gamma_L^+)^{1/2} + (\gamma_S^+ \gamma_L^-)^{1/2} \}$$

where γ is the total surface energy, γ^{LW}, γ^+ and γ^- are respectively the van der Waals, electron-acceptor and electron-donor components of the surface energy, θ is the contact angle and the subscripts S and L are relative to the solid (sample) and liquid.

Atomic force microscopy (AFM)

The microscope was a Topometrix 2010 "Discoverer" working in contact mode, with a liquid cell and silicon nitride tips. After rinsing, the samples were transferred to the cell, which was filled with the transportation water. All observations presented here were performed under water. The scan speeds were of the order 10 µm/s and the applied force was the minimum possible (~1nN).
Table 3 summarises all information concerning the characterisation techniques

Results and discussion

Reference samples

The alloy surface is covered with a thin (Fe,Cr) oxide film (a few atomic layers) mainly composed of Fe^{3+}_{ox} and Cr^{3+}_{ox} already characterised in previous work (Costa *et al.*, 1998). The composition of this surface oxide does not significantly change upon short immersion in seawater (up to 24 hours). The presence of an organic contamination layer on the alloy surface after the cleaning procedure using acetone and ethanol is unavoidable. Figure 1a shows the C 1s signal of the XPS spectrum obtained from one sample after surface preparation. This signal contains several components: an intense signal at 285 eV ascribed to carbon from C-C and C-H bonds, shoulders at 287-288 eV from carbon in C-O, C=O, C-N or C=N bonds, and one small peak at 289 eV from carbon in O-C=O groups. The C-N or C=N signals are certainly very weak since only a small signal in the N 1s region, Figure 1b, is observed on a sample immediately after cleaning. The nitrogen peak is centred at 400.2 eV, it is broad and could include the contributions of the C=N, C-N and N-H functions. A small amount of contamination due to the presence of NO is also revealed in Figure 1b.
Figure 2 shows an AFM image of the dry surface of a sample after cleaning but before immersion. A central zone is visible, which had been previously scanned by the tip at high contact force. The surface material has been swept from this zone and has accumulated around the edges. The maximum thickness of this surface contamination is evaluated at around
0.5 +/- 0.2 nm.

It is well known that stainless steel surface energies are strongly dependent on the preparation procedure and the cleaning treatment (Boulangé-Petermann *et al.*, 1993). Table 4 gives the contact angles measured with three pure liquids on stainless steels with or without cleaning with acetone and ethanol and the corresponding energetic characteristics of the samples. Without any cleaning with a solvent, the electron donor values, γ^-, are smaller. Thus the surface contamination left after a simple rinsing in water yields more hydrophobic surfaces. Comparable but slightly lower values of surface free energies were obtained by Boulangé-Petermann *et al.* (1993) on AISI 304L after degreasing with organic solvents which do not eliminate all the organic matter contaminating stainless steel surfaces.

Conditioning layer adsorption as a function of immersion time

The presence of a conditioning layer is detected by the different analytical techniques used. Figures 3 and 4A show the N 1s and C 1s region spectra for increasing times of immersion during June 97. The nitrogen peak intensity, Figure 3 and the high-energy C 1s signal increase with longer immersion time. In contrast, the C 1s signal at lower energy, Figure 4A, does not follow any systematic trend with immersion time. Though rather weak, this evolution appears in Figure 4B, in which curves α and β present the high-energy signals from samples immersed for 5 and 24 hours respectively after subtraction of the signal from a polished sample. A signal located at 286.2 ± 0.2 eV appears, corresponding to C-O, C-N or C=N bonds (C $1s^{org}$ signal) in both spectra. Small signals at 287.0 eV and 289.4 eV corresponding respectively to C=O and O-C=O or C=O-NH from a peptidic bond are also observed in Figure 4Bα, after 5 hours of immersion. Figure 5 presents the evolution of both N 1s and C $1s^{org}$ at 286.2 eV with immersion time and makes clear an increase in the amount of organic species on the surface.

The corresponding IRAS spectra obtained under identical conditions are shown in Fig. 6 and will help to characterise the chemical nature of the adsorbed species.

The figure shows the IRAS spectra of stainless steel samples after 5 and 24 hours of immersion. Chemical functional groups of biomolecules can be identified on these spectra . After 5 hours, bands at 1520 and 1680 cm^{-1}, ascribed to respectively the N-H bending, C-N stretching (amide II band) and C=O stretching (amide I band) vibration modes, suggest the adsorption of proteins on the surface. The signal at 1250 cm^{-1}, called the amide III band by some authors (Nivens and Schmitt, 1993) who attribute it to an amide C-O stretch, is in fact more probably the P=O stretching vibration from organophosphorous and could indicate the presence of lipids as already suggested by Taylor (1997).This assumption is confirmed by the presence a small phosphorous peak detected in the XPS overview spectrum. Fatty acids (essentially from phytoplankton degradation) may account for approximately 4% of the dissolved organic carbon in natural waters (Thurman, 1985). After 24 hours of immersion, in addition to the remaining bands at 1520 and 1680 cm^{-1}, the spectrum is now dominated by a triple-peak at 1050-1120-1180 cm^{-1}, characteristic of C-O, C-O-C bonds.

The increasing surface concentration of compounds containing C-N bonds on the one hand, and C=O and C-O bonds on the other, is in agreement with the evolution of the C $1s^{org}$ signal and N 1s signals of the XPS data. Thus, both techniques, XPS and IRAS suggest successive adsorptions of two different types of species; amine functional groups, which are likely to originate from proteins, followed by carbohydrates. The latter species are dominant on the surface after 24 hours of immersion. It will be shown later that the adsorption kinetics may be influenced by the surface preparation method. Note that we have neglected any preferential orientation of functional groups which would greatly influence the IR signal intensities, since only vibration modes normal to the surface are active in IRAS. We assume a random orientation of the chemical groups in the adsorbed phase. This may not, however, be completely true for polysaccharides, which are known to be more " deformable " (Boshle *et al.*, 1998) than proteins and which consequently may adopt a partially ordered structure when interactions exist with the surface. This could explain the rather high intensity of the C-O, C-O-C bands. After staining of the samples with a fluorescent molecule (DAPI), the total number of bacteria was estimated using epifluorescence microscopy. Due to the very small quantity of bacteria attached to the surface after 24 hours of immersion ($\leq 10^3$ $/cm^2$), we may neglect their contribution to the XPS and IRAS results.

Some differences also appear in the surface energy characteristics of two samples as a function of immersion time during June 1997. Table 5 shows that for one sample, the electron-donor component γ^- of the surface energy increases after 5 or 24 hours of immersion, while no change is observed for the other sample in comparison with the results obtained on a "clean" surface (Table 4). Nevertheless, considering the observed standard deviations, these modifications essentially show an increase in the hydrophilic character γ^- with immersion time in natural seawater. Schneider (1997) showed, from water contact-angle measurements, that the wettability of clean hydrophobic surfaces was generally increased and that of hydrophilic surfaces decreased after one hour of immersion in static filtered seawater (0.1µm). These results are in accordance with our measurements on conditioned surfaces in circulating natural seawater. The discrepancies between the electron-donor components γ^- of the two samples (Table 5) may be due to significant surface heterogeneities.
Figure 7 shows AFM images obtained on stainless steel surfaces after 0, 5, and 24 hours of immersion in natural seawater in June 1997. The images were obtained during *in situ* measurements in sea water using conditions under which the tip did not displace the particles. In all cases, the deposit is extremely heterogeneous. After a 5h immersion in summer, there is a significant deposit in the form of particles present on the surface (Figure 7b). After 24 hours, in some areas, the coverage is sufficiently dense to be almost considered as a locally continuous film (Figure 7c). The overall coverage increases with immersion time, but some regions remain bare, while others are covered with particles of varying size —see figure 8. But the general trend of increasing coverage with time seems fairly clear. This may explain the large variations obtained by Taylor *et al.* (1997) in conditioning layer thicknesses measured by optical ellipsometry on stainless steel samples after 3 days of immersion in oligotrophic, subtropical waters (120 ± 110 nm). It is difficult to evaluate the average thickness of this conditioning layer due to its heterogeneity, but on the covered area, it is estimated at a few nanometers after 24 hours of immersion. Surface heterogeneities revealed by both the AFM images and γ^- measurements are confirmed by XPS and ToF-SIMS data. Metal signals (Fe 2p, Cr 2p peaks and Fe^-, Cr^- ions) are still detected even after 24 hours of immersion in spite of a large total amount of carbon on the surface (Pradier et *al.*, 2000). This heterogeneity, which has already been suggested by a few authors (Taylor et *al*.,1997) was mentioned in our previous work (Pradier *et al.*,2000) and is confirmed by these results. It could explain why, even in the presence of this primary adsorbed layer, the substratum properties may still play a role in bacterial adhesion as suggested by Baier (1980).

Effect of sample cleaning on subsequent adsorption

A new rinsing procedure using hexane has been tested. In July 1998, ethanol rinsing was replaced by hexane rinsing before immersion in natural seawater. Some complementary IRAS, XPS spectra and surface energetic measurements were also recorded after rinsing in ethanol or hexane followed by immersion in September 98 in order to see whether the rinsing procedure also affects the binding of molecules upon immersion in seawater. Whatever the cleaning procedures are, the presence of an unavoidable organic contamination layer left on the alloy surface is revealed by analytical techniques such as XPS and ToF-SIMS. And even if cleaning with organic solvents reduces the contamination layer, the air-contact after the treatment will always produce organic contamination on the sample surfaces, as noted by Rouxhet (1990). The comparison of ToF-SIMS measurements after rinsing in ethanol or hexane shows that the major contaminants of the surface in the first case are hydrocarbons (especially PDMS) which are significantly reduced after hexane cleaning. PDMS is a very common surface contaminant owing to its very low surface free energy and is present in

numerous laboratory atmospheres. The ToF-SIMS spectra from positive and negative ions obtained on hexane-rinsed substrata are shown in Figure 9. The main peaks in the figures are due to the isotopes of Fe and Cr for the positive ions and those of O and OH for the negative ones. In addition to the contamination by hydrocarbons, peaks related to inorganic elements (Na^+, Cs^+, NO_3^-, PO_2^-, PO_3^- , SO_3^-, F^- et Cl^-) and fatty acids (227^-, 255^- and 283^-m/z) are also detected. However, ToF-SIMS measurements show that the contamination of the surface by silicon (whose peak masks all the other signals) is significantly reduced using hexane rinsing in comparison with ethanol. Thus, the adsorption of the conditioning layer is more easily detected after hexane rinsing. It is not possible to say here whether the PDMS itself influences the formation of the conditioning film. The adsorption of this layer is evidenced by the presence of and the increase in some secondary ion peaks in ToF-SIMS measurements: NH_3^+, NH_4^+, CH_3O^+, $C_5H_{12}N^+$, $C_8H_5O_3^+$ for the positive ions and NH^-, CN^-, CNH^-, CH_3O^-, O_2^-, C_2O^- , CNO^-, $CNOH^-$, $COOH^-$, HNO_2^- for the negative ions. Figure 10 shows the positive (a) and negative (b) spectra obtained after 24 hours of immersion in natural seawater in July 1998. The higher intensities correspond to the isotopes of Fe, Cr and Ni in the positive mode and O^- and OH^- in the negative mode. And as previously observed on a reference sample, contamination by hydrocarbon compounds is still present. Peaks related to inorganic ions (Na^+, Mg^+, Cs^+, F^-, Cl^-, NO_3^-, PO_2^-, PO_3^-, SO_3^-, SO_4^-, HSO_4^- and I^-) and to fatty acids (227^-, 255^- and 283^- m/z) are still detected. The evolution of the relative intensities of specific positive ions (a=NH_4^+, b=CNO^- and c=$C_5H_{12}N^+$) related to proteins and of specific negative ions (a=C_2O^- and b=$COOH^-$) related to carbohydrates is shown as a function of immersion time in Figures 11 and 12. These data are obtained from stainless steel samples after hexane rinsing and are estimated using Equation 1. The relative intensities of ions given at t=0 and 0.5 hours correspond to those detected on the reference and on the "0 hour immersion" samples respectively**.** The intensities of the signals attributed to NH_4^+,CNO^- and $C_5H_{12}N^+$ species increase rapidly for the first 8 hours, then more slowly, to reach a plateau after around 24 hours of immersion. The same behaviour is observed for the ions NH_3^+, NH^-, CN^-, CNH^-, NO^-, $CNOH^-$, HNO_2^- and confirms a very rapid adsorption of amines on the surface. Ions related to inorganic species also follow the same pattern (B^+, Na^+, Mg^+, Ca^+, Ti^+,Br^-, PO_x^- and SO_x^- and I^-)

An increase with immersion time of the relative intensities of negative ions (a=C_2O^- and b=$COOH^-$) and of such ions as CH_3O^-, O_2^-, CH_3O^+ is also revealed. However, the intensities of these peaks are generally higher for the reference samples due to the surface contamination by hydrocarbons after the cleaning procedure. After one hour of immersion, a progressive cleaning of the surface seems to occur, leading to a decrease in the intensities of the peaks linked to C-O, C=O species, then an adsorption of carbohydrates is observed, up to a quasi-steady state value at around 24 hours of immersion.

Figure 13 shows ToF-SIMS measurements of the evolution with time of peaks related to two typical ions in stainless steel : Cr^- and Fe^-. The peak intensities decrease to a limiting value reached after 8 hours of immersion and do not tend towards zero even after 24 hours. Similar results are obtained for the Fe + Cr XPS signals. Even after 24 hours of immersion the peaks due to the metallic contribution of the substratum (Fe, Cr) are still visible (Pradier *et al.*, 2000), showing that this layer is not uniform but discontinuous. From the attenuation of the metal signals observed by XPS and ToF-SIMS, and assuming that only the uncovered surface contributes to these signals, the surface coverage is estimated at around 50 % of the total surface after 24 hours of immersion in July 1998.

IRAS spectra are slightly different for immersions performed after rinsing in hexane rather than in ethanol. This "solvent" effect is clear from Figures 14a and b comparing IRAS spectra obtained with the two cleaning procedures before immersion in September 1998.

Bands appear at similar wave-numbers in this figure, indicating, at first glance, the growth of similar organic layers, but some differences are worth noting. The intensities of the main bands are, for equal times of immersion in September 1998, four times higher on samples rinsed in hexane as compared to those rinsed in ethanol. The main difference observed in the IRAS spectra between the two cleaning procedures lies in the 1510 cm^{-1} band, which is hardly detectable for short times of immersion when the other signals are still weak after hexane cleaning but clearly seen in Figure 14a after ethanol cleaning. During the initial steps of immersion after an ethanol rinsing (spectra after 5h and 8h in Figure 14a), the bands at 1510 cm^{-1}, and 1050-1100 cm^{-1} could be the fingerprints of secondary amines adsorbed on the alloy surface. These two bands become negligible after long times of immersion, for which the dominant band is at 980-1020-1050 cm^{-1}. The latter can certainly be ascribed to ν(C-O) in
C-O-C groups or ν(C-OH) characteristic of carbohydrates. In addition to this broad absorption signal, two bands at 1400 and 1600 cm^{-1} appear after 24 hours of immersion and can be attributed to the symmetric and asymmetric stretching modes of CO_2^- groups of acids present in some carbohydrates bound to humic substances. These two bands also show up in the spectrum of Figure 14b corresponding to 24 hours of immersion, but they are relatively much weaker. Such a variation in the relative band intensities is often encountered in reflection infrared measurements. Finally, the band in Figure 14a, at 870 cm^{-1}, which increases with immersion time, is attributed to the presence of sulphates (such as $MgSO_4$).
It is interesting to compare the two series of IR data obtained on samples cleaned in ethanol in June 97, Figure 6, and September 98, Figure 14a. Spectra show vibration bands at similar positions although, in the latter data, the bands at 1510 disappeared after 24 hours of immersion, suggesting a replacement of the initially bound proteins by carbohydrates. These differences observed for short times of immersion (2 hours) also appear in the N 1s and C $1s^{org}$ signals of XPS spectra as a function of cleaning procedure, as illustrated in Figure 15, which also shows that the adsorption of N-containing species seems to be slightly delayed with hexane cleaning.
In summary, rinsing the samples in hexane rather than in ethanol seems to delay nitrogen-containing species adsorption during the initial stage of surface coverage. But XPS results show that after 24 hours of immersion, the adsorbed quantities of organic matter on the surface are the same after hexane or ethanol rinsing (Figure 15). XPS cannot confirm the greater amount of adsorbed organic species detected by IRAS after rinsing with hexane due to the limited depth of analyses (~50 Å), Table 3.
The surface-energetic characteristics are slightly different when immersions are performed after rinsing in hexane, as is shown in Table 6 and Figure 16, in which all the data are from the same period of immersion (September 1998) but using the two different cleaning procedures. The increase in the γ^- component is more marked after rinsing with hexane. However, the surface energies of samples cleaned with hexane and ethanol were already somewhat different before immersion in natural seawater, as shown in Table 6. The hydrophilic character was already more pronounced on a hexane rinsed sample before immersion in seawater. Nevertheless, whatever the cleaning procedure, the increase in the hydrophilic character γ^- with immersion time in natural seawater previously mentioned is again confirmed. All these results show the influence of the sample preparation method on conditioning layer formation.

Conclusions

Using complementary surface analytical techniques, the chemical composition and the growth kinetics of the adsorbed layer on stainless steel during the period immediately following

immersion in natural seawater have been determined. XPS, ToF-SIMS and IRAS measurements clearly show that successive adsorptions of two different types of compounds occur; nitrogen-containing species (probably proteins) in the first stage and carbohydrates in the second. This adhesion occurs before any bacterial attachment. An increase in the amount of organic species adsorbed is observed with time up to 24 hours of immersion. Even after 24 hours of immersion, no continuous film is present on the stainless steel surface; heterogeneities revealed by both AFM images and surface free energy parameters are confirmed by XPS and ToF-SIMS data. This could explain why, even in the presence of this primary adsorbed layer, the substratum properties may still play a role in bacterial adhesion, as is suggested by a few authors. The effect of different cleaning procedures (acetone and ethanol or hexane) on the subsequent adsorption of organic molecules on a surface after immersion in natural seawater is important. The cleaning method influences the chemical composition of the first layers of molecules adsorbed after immersion .

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Table 1: Exposure periods and surface preparation

	Rinsing		Seawater T°C
June 1997	Ethanol		15.3
September 1998	Ethanol	Hexane	16.1

Table 2: Liquid/vapour surface tension parameters of the liquids γ^{LW} : van der Waals component, γ^{+} : electron-acceptor component, γ^{-} : electron-donor component from Van Oss *et al*. except γ^{LW} of α-Bromonaphthalene liquid measured at INRA.

Liquid	Liquid-energetic values (mJ/m^2)		
	γ^{LW}	γ^{+}	γ^{-}
Water	21.8	25.5	25.5
Formamide	39.0	2.3	39.6
Diiodomethane	50.8	0	0
α-Bromonaphthalene	40.0	0	0

Table 3: Information concerning the characterisation techniques and the analyses.

	Depth of analysis (Å)	Spot size (µm)	N° of samples
ToF-SIMS	< 5	2 (diameter)	4 random areas analysed on one sample surface
XPS	50-100	2000 x 6000	2
IRAS	< 10000	5000 x 1000	3
AFM	< 10	5 to 10.10^{-3} (diameter)	3 random areas analysed on two sample surfaces. Images acquired at 3 magnifications in each analysed zone
Contact angles	< 5	*	3 random areas analysed on two sample surfaces.

* not applicable

Table 4: Contact angles measured with three pure liquids and surface-energetic values of AISI 316L stainless steel samples as a function of cleaning procedures (± denotes the standard deviation)

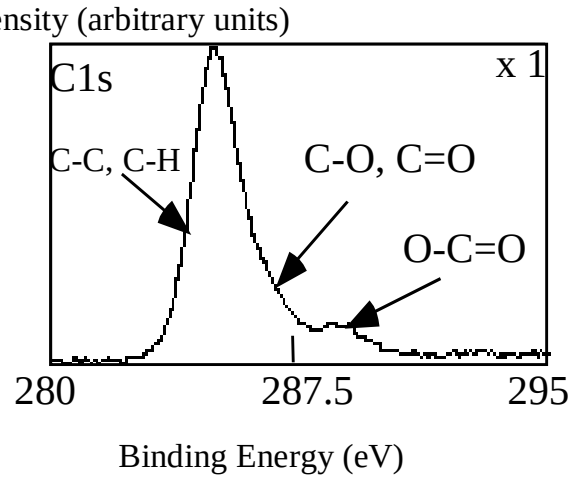
	Contact angles (degrees)			Surface-energetic values (mJ/m^2)		
	θ_{water}	$\theta_{formamide}$	$\theta_{diiodomethane}$	γ^{LW}	γ^{+}	γ^{-}
Sample polished and rinsed with demineralised water	67±2	42±6	44±1	37.4	1.4	10.4
Sample polished and rinsed with acetone and ethanol	60±4	43±2	41±5	39.2	0.5	18.9

Table 5: Contact angles measured with three pure liquids and surface-energetic values of two AISI 316L stainless steel samples as a function of immersion time in natural sea water in June 1997 (± denotes the standard deviation).

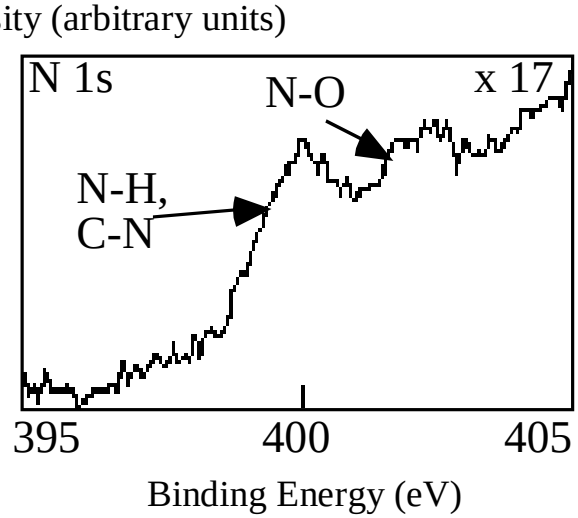
Immersion time (h)	Contact angles (degrees)			Surface-energetic values (mJ/m^2)		
	θ_{water}	$\theta_{formamide}$	$\theta_{\alpha\text{-bromonaphtalene}}$	γ^{LW}	γ^{+}	γ^{-}
	51±3	48±1	22±2	37.1	0.1	34.3
5						
	68±5	53±3	35±2	33.1	0.6	14.6
	70±6	54±5	33±2	33.8	0.5	13.0
24						
	58±1	48±1	31±3	34.5	0.5	24.0

Table 6: Contact angles, measured with three pure liquids, of AISI 316L stainless steel samples as a function of immersion time in natural sea water in September 1998 after different cleaning procedures (± denotes the standard deviation).

Immersion time (h)	Contact angles (degrees)					
	Cleaning with acetone and ethanol			Cleaning with hexane		
Immersion time	θ_{water}	$\theta_{formamide}$	$\theta_{diiodomethane}$	θ_{water}	$\theta_{formamide}$	$\theta_{diiodomethane}$
0	66±2	56±1	47±2	59±1	52±1	44±1
5	56±1	47±1	44±1	37±2	36±6	50±1
24	58±3	52±1	39±2	41±2	31±5	35±1



a



b

Figure 1: XPS spectra of sample after cleaning procedure. a: C1 s signal b: N1 s signal.

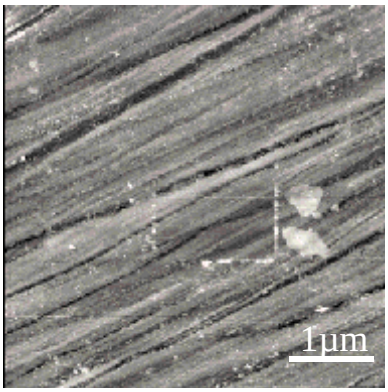
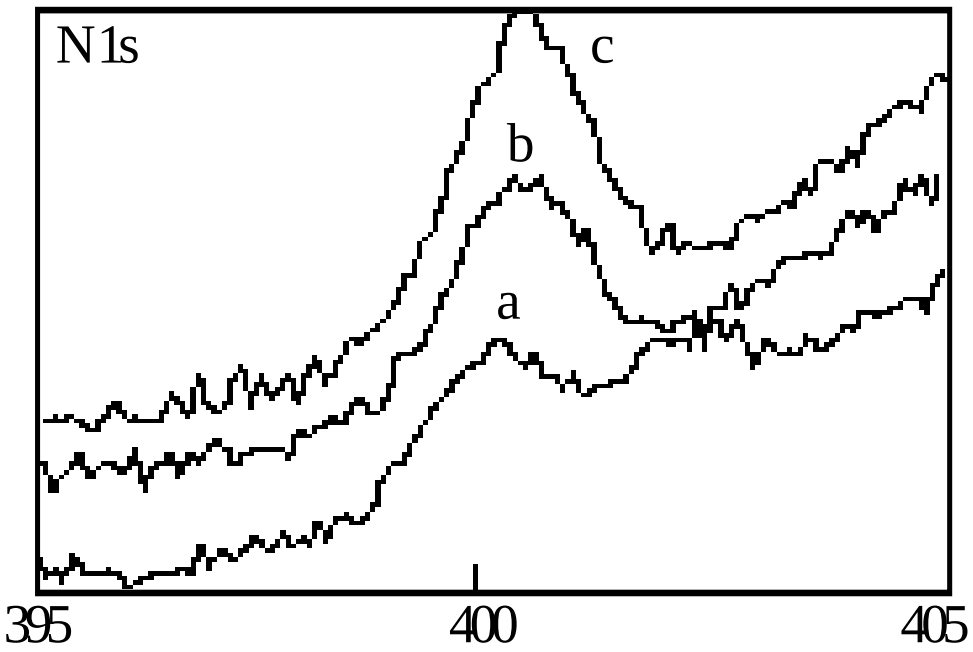


Figure 2: AFM image of the dry surface of a sample after cleaning procedure.

Intensity (arbitrary units)



Binding Energy (eV)

Figure 3: N 1s region spectra for increasing times of immersion during June 97. a: Polished sample, b: 5 hours immersion, c: 24 hours immersion.

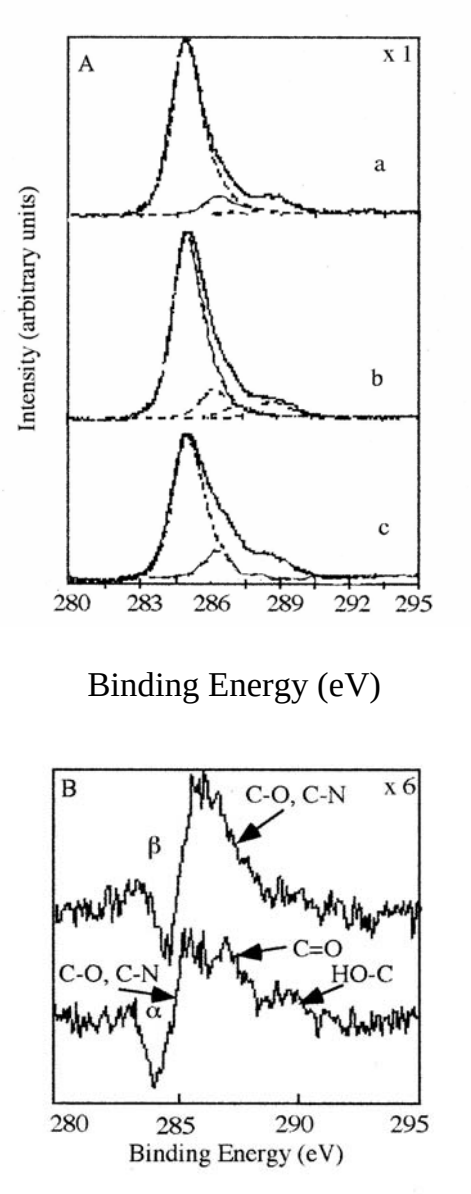


Figure 4: A: XPS spectra of the C1s regions recorded after a: sample polishing (reference sample), b: 5 hours and c: 24 hours of immersion in natural seawater. The spectra are fitted with 4 contributions, C-C, C-H (285 eV), C-O, C-N (286.2 eV), C=O (287.0 eV) and HO-C (289.4 eV). B: C1s high-energy signals obtained by subtraction of the C-C, C-H contribution to signals b and c, α: (b-a) : (5 hours - polished sample) and β: (c-a) : (24 hours - polished sample) spectra.

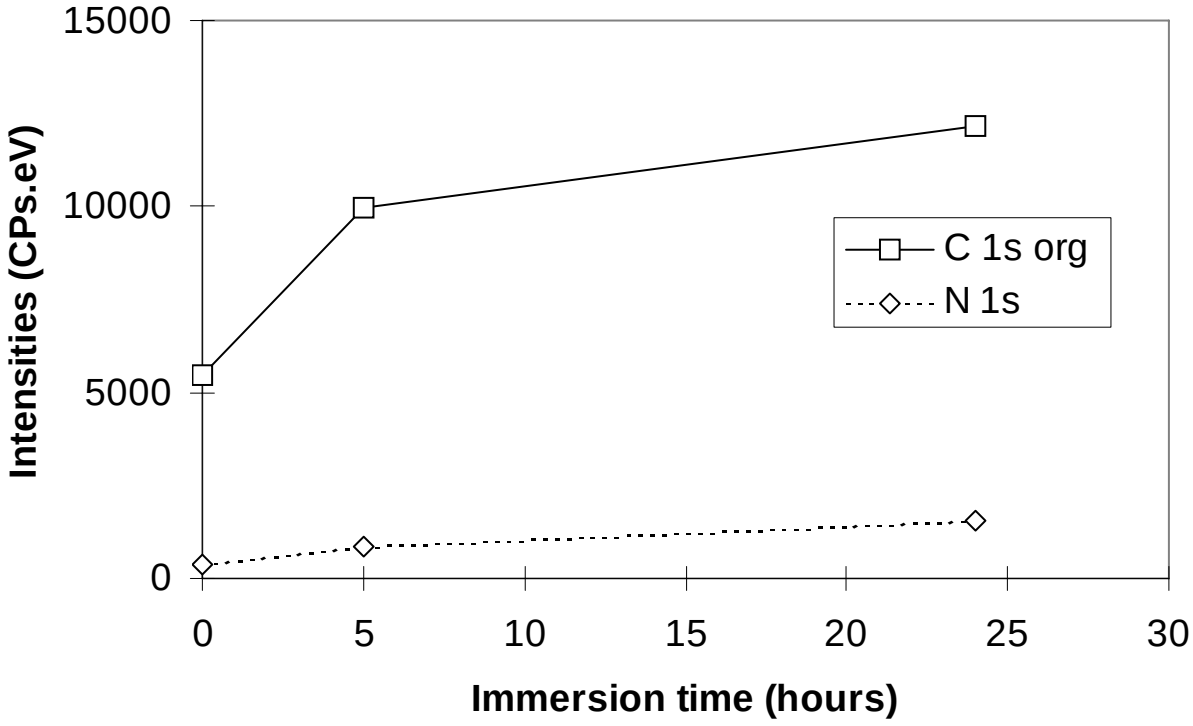


Figure 5: Evolution of both N 1s and C $1s^{org}$ with immersion time in natural seawater in June 1997.

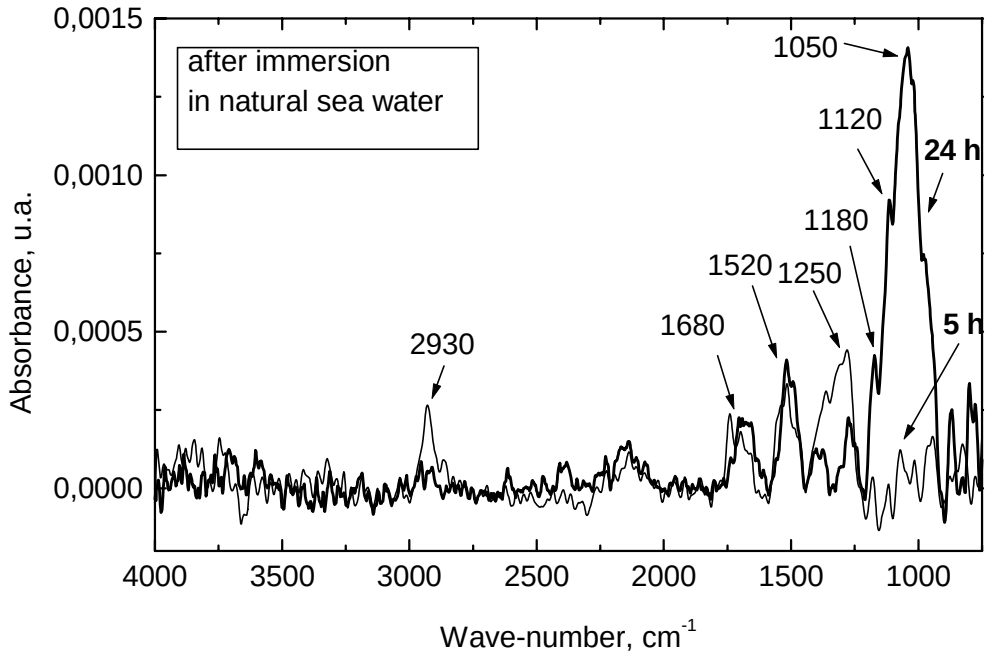


Figure 6: Evolution of IRAS spectra with immersion time in natural seawater during June 1997. Time of immersion 5h (thin line), 24 h (thick line).

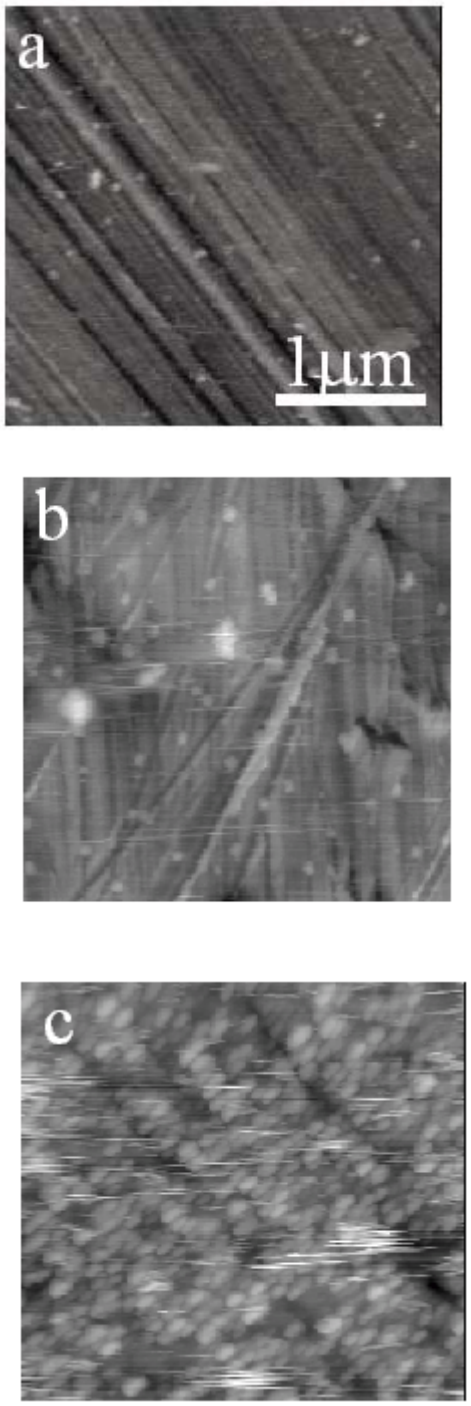


Figure 7: Evolution of stainless steel surface with immersion time in June 1997 :

a = 0h, b = 5h, c = 24h. (Magnification is the same for all AFM images).

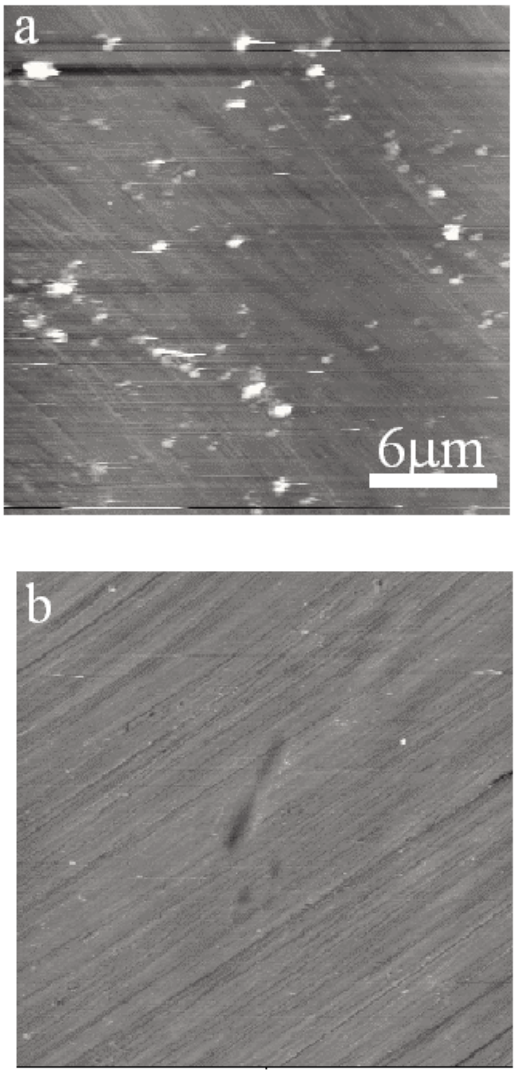


Figure 8: Two regions exposed for 24h in nominally identical conditions in June 1997

(Magnification is the same for both AFM images)

a

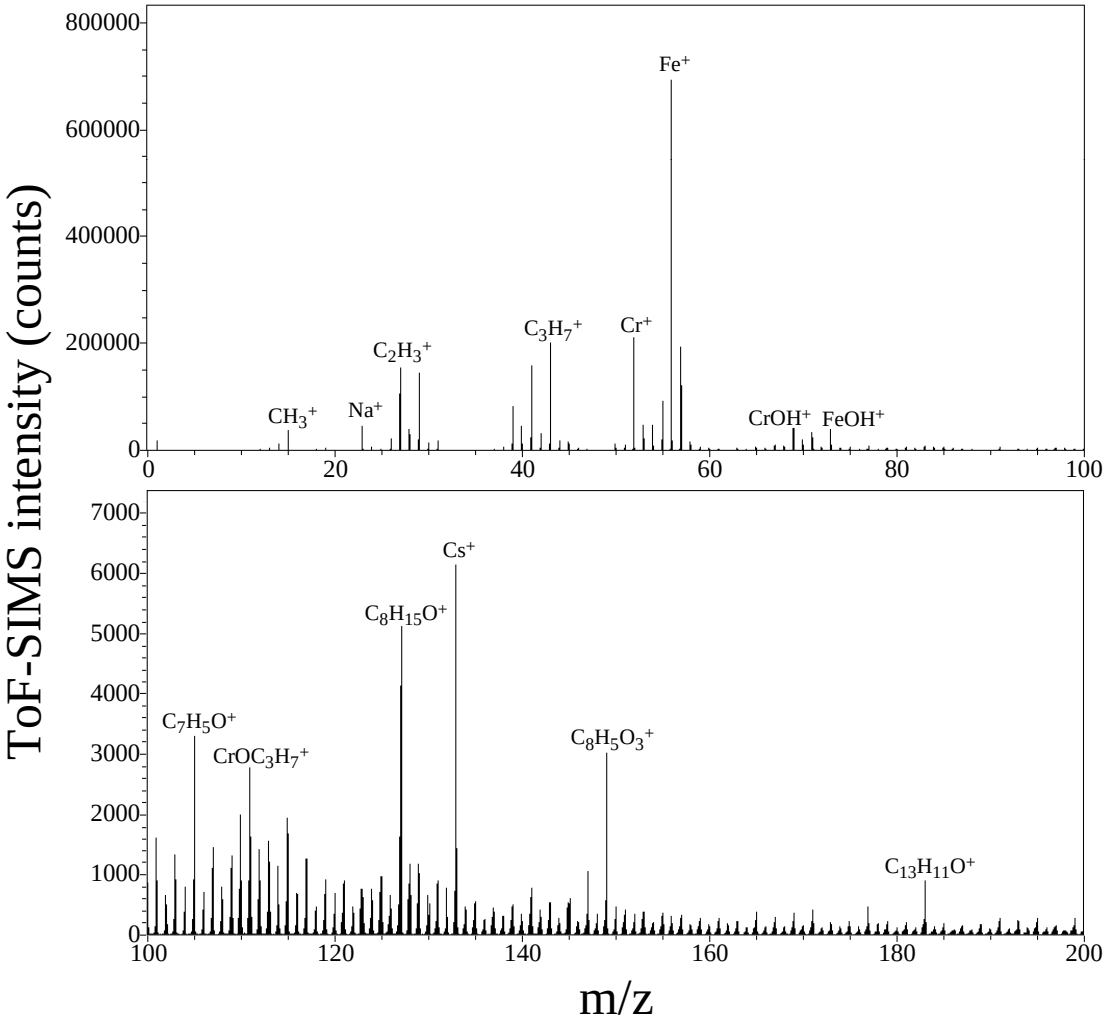


Figure 9-a: Positive ToF-SIMS spectra from stainless steel after hexane rinsing.

b

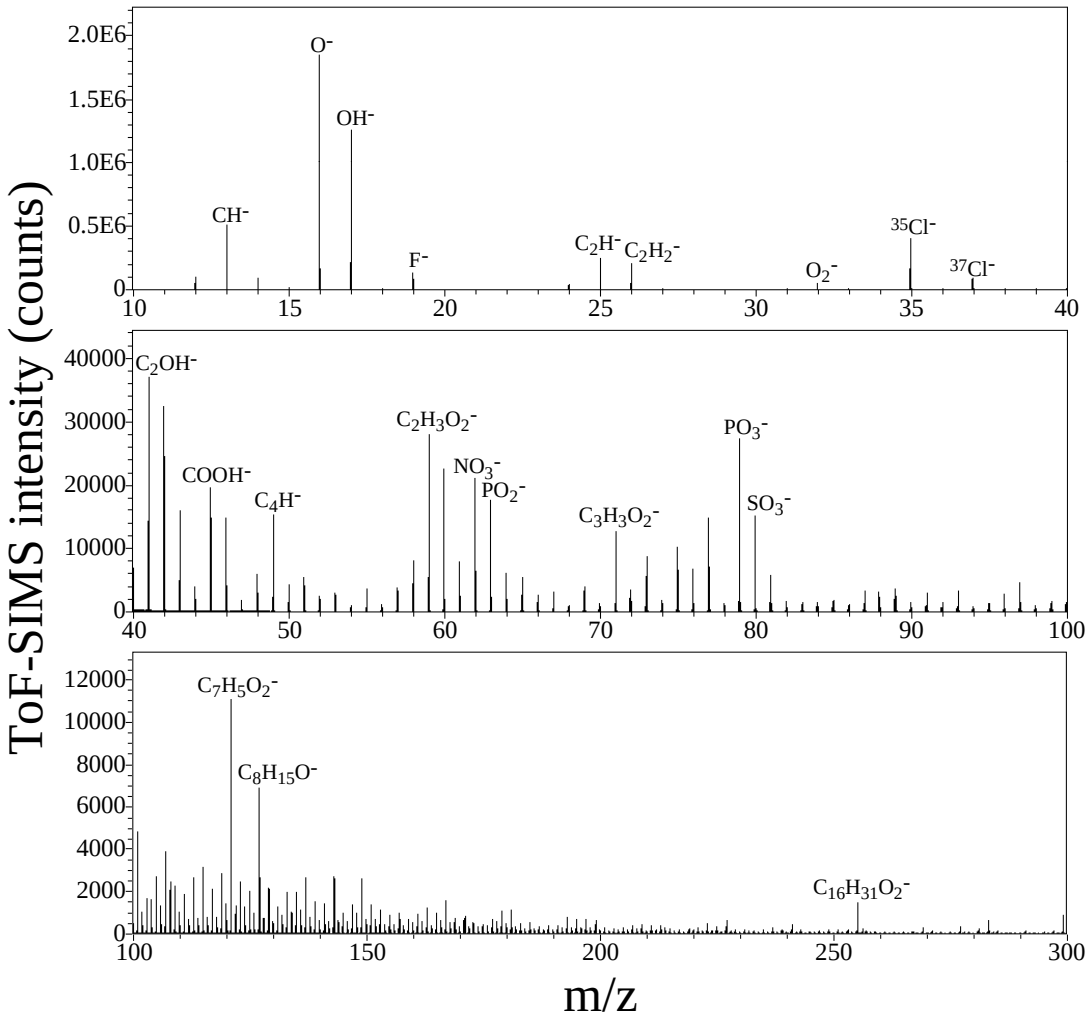


Figure 9-b: Negative ToF-SIMS spectra from stainless steel after hexane rinsing.

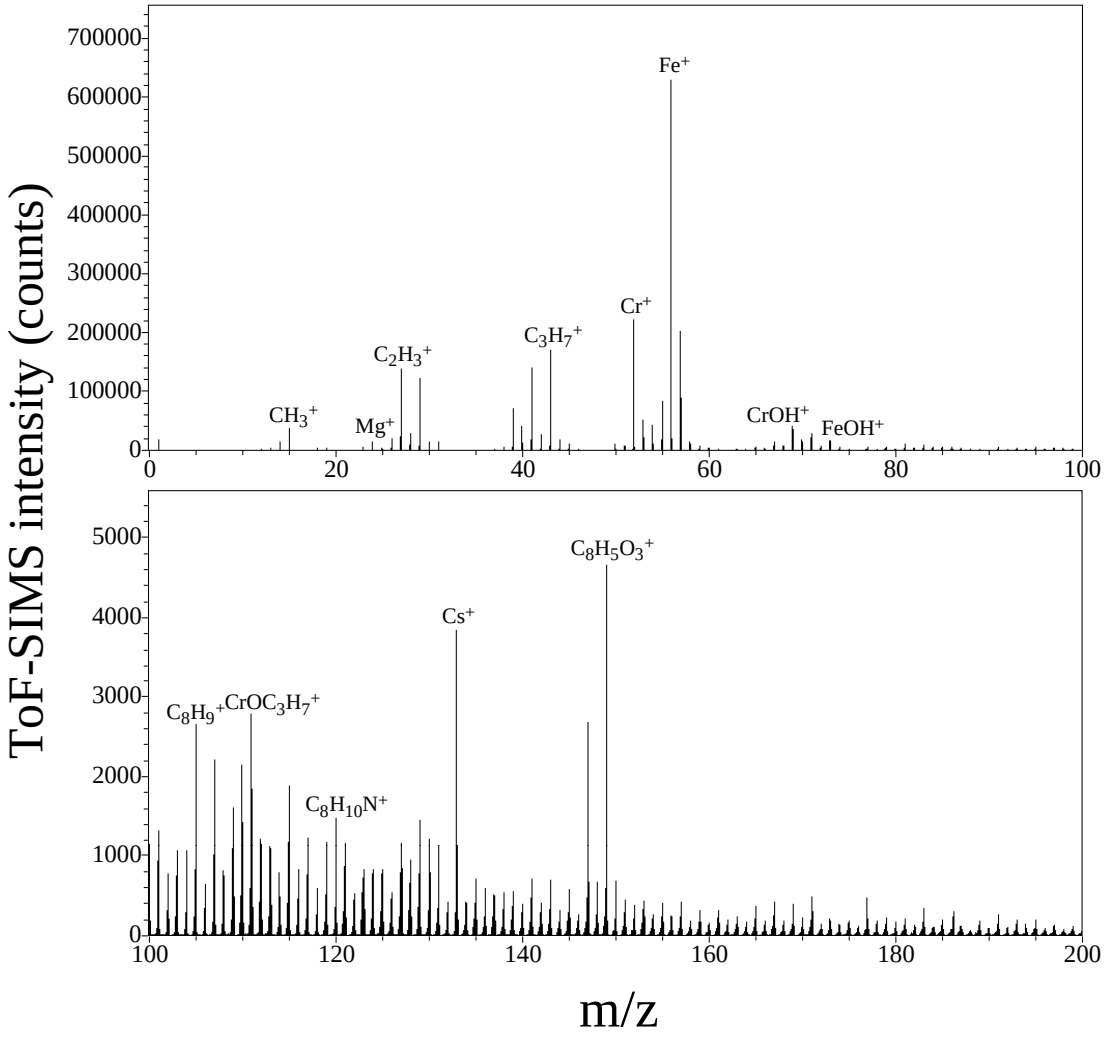


Figure 10-a: Positive ToF-SIMS spectra obtained after 24 hours of immersion in natural seawater in July 1998.

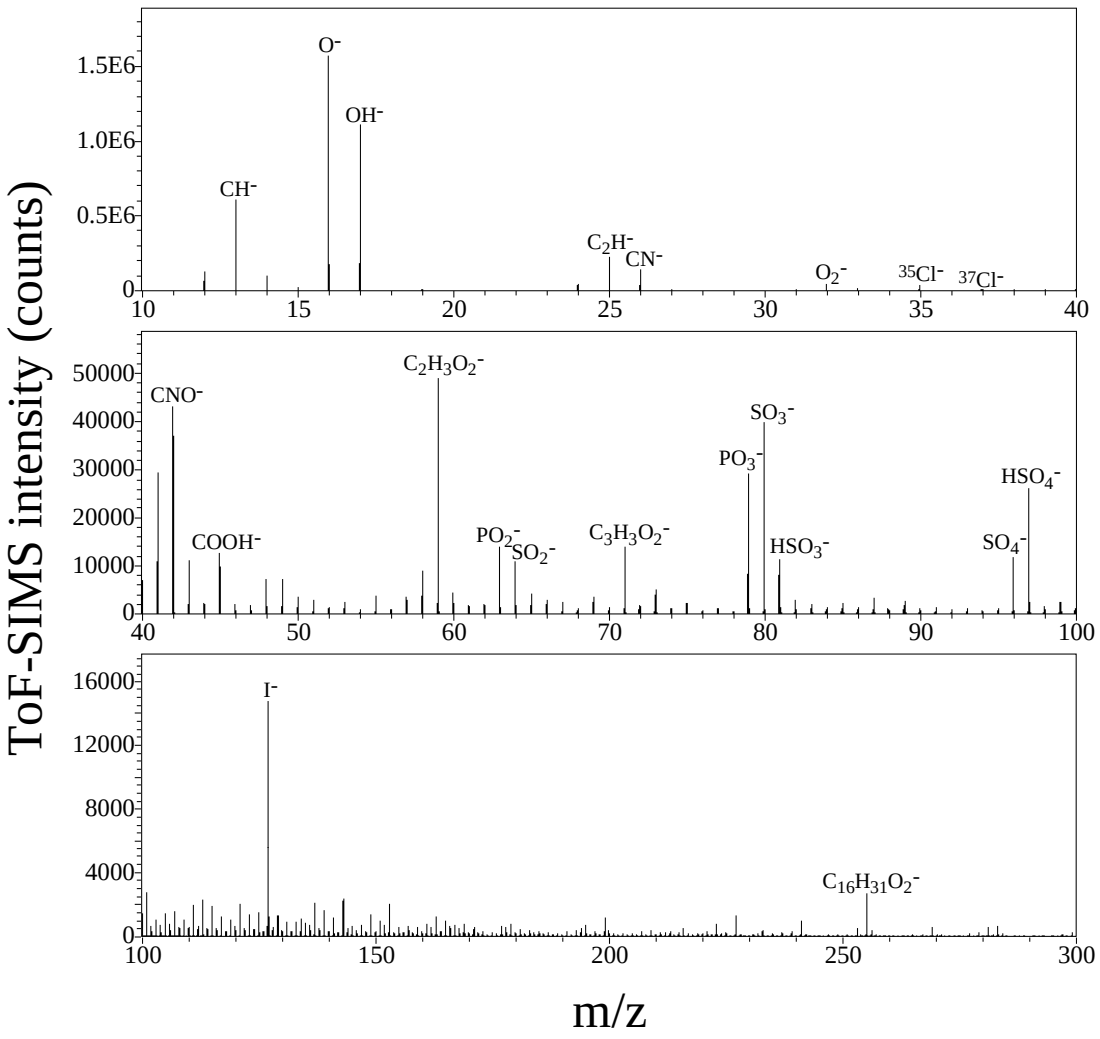


Figure 10-b: Negative ToF-SIMS spectra obtained after 24 hours of immersion in natural seawater in July 1998.

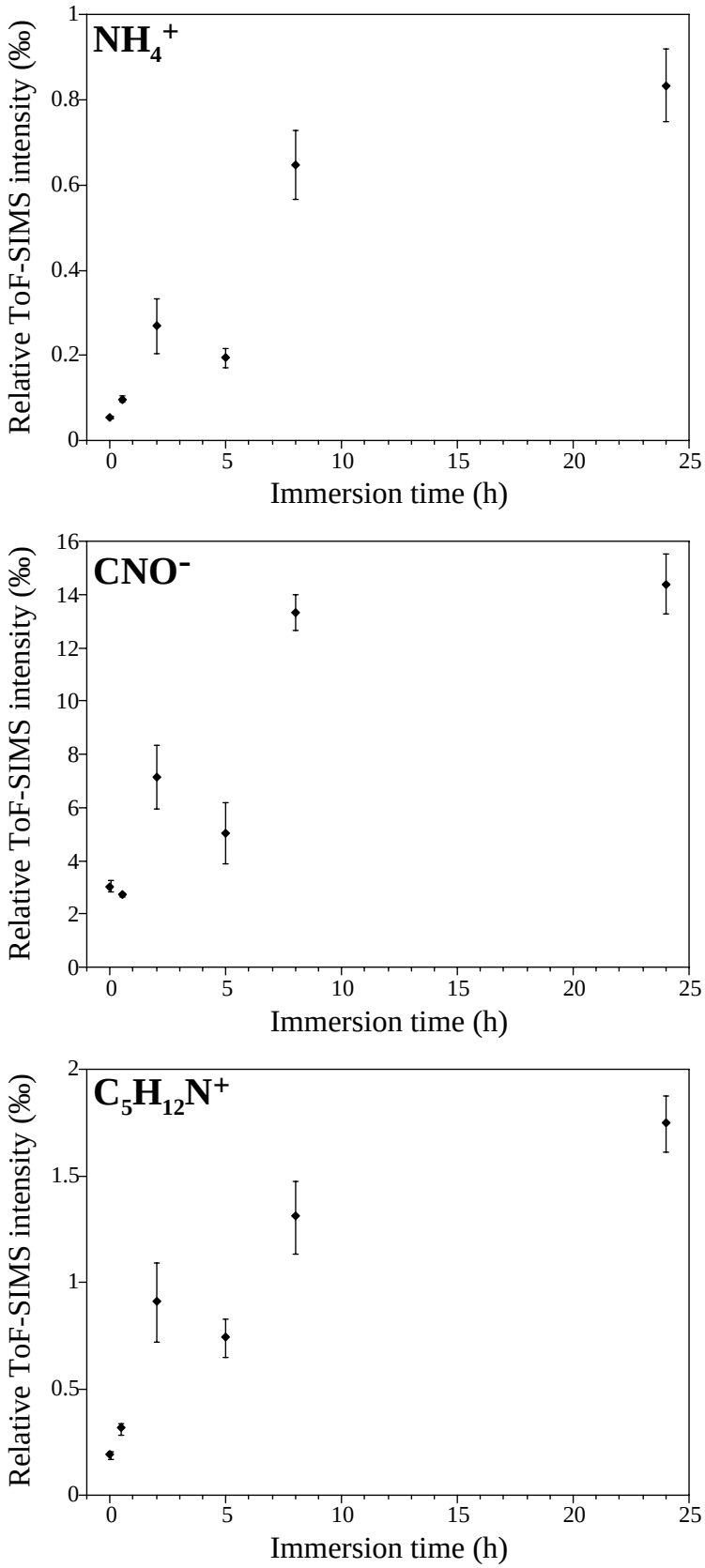


Figure 11: Evolution with time of ToF-SIMS peaks related to proteins : NH_4^+, CNO^- and $C_5H_{12}N^+$.

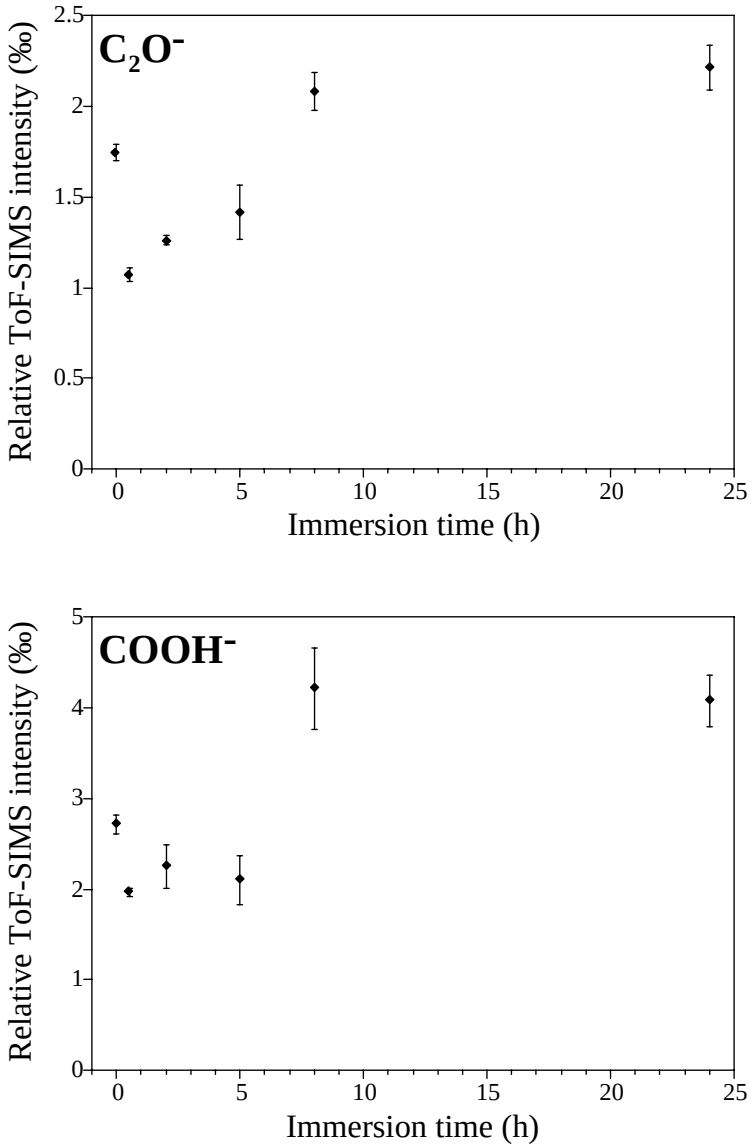


Figure 12: Evolution with time of ToF-SIMS peaks related to carbohydrates: C_2O^- and $COOH^-$.

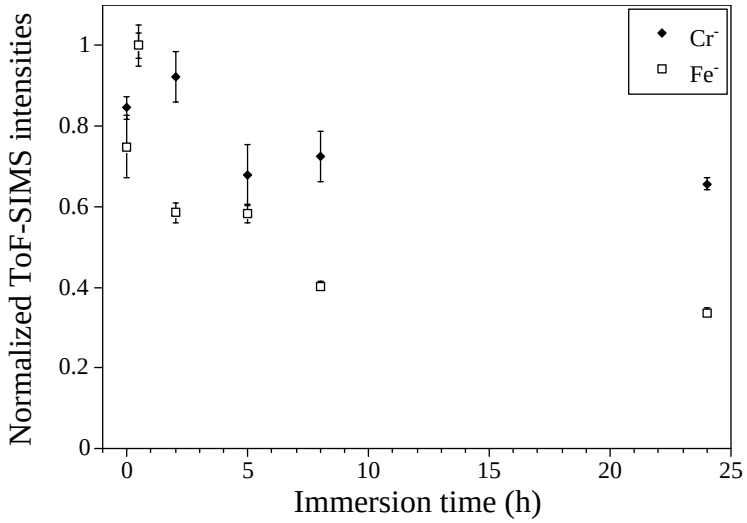


Figure 13: Evolution with time of ToF-SIMS peaks related to two typical ions in stainless steel: Cr^- and Fe^-.

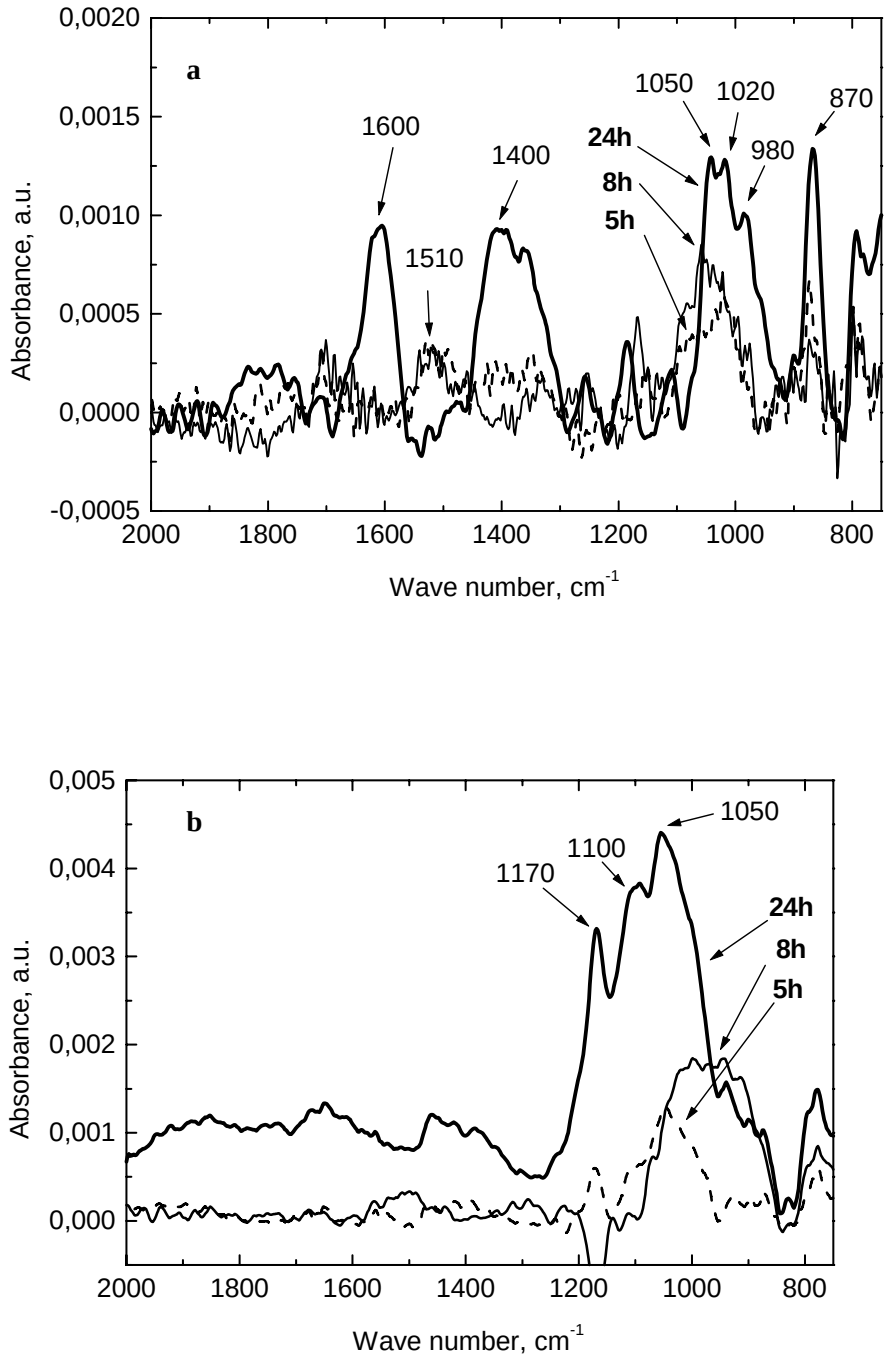


Figure 14: IRAS spectra obtained on stainless steel samples as a function of immersion in natural seawater in September 1998 after a cleaning procedure with acetone and ethanol (a) or with hexane (b). Time of immersion : 5h (dot line), 8h, (thin line), 24 h (thick line).

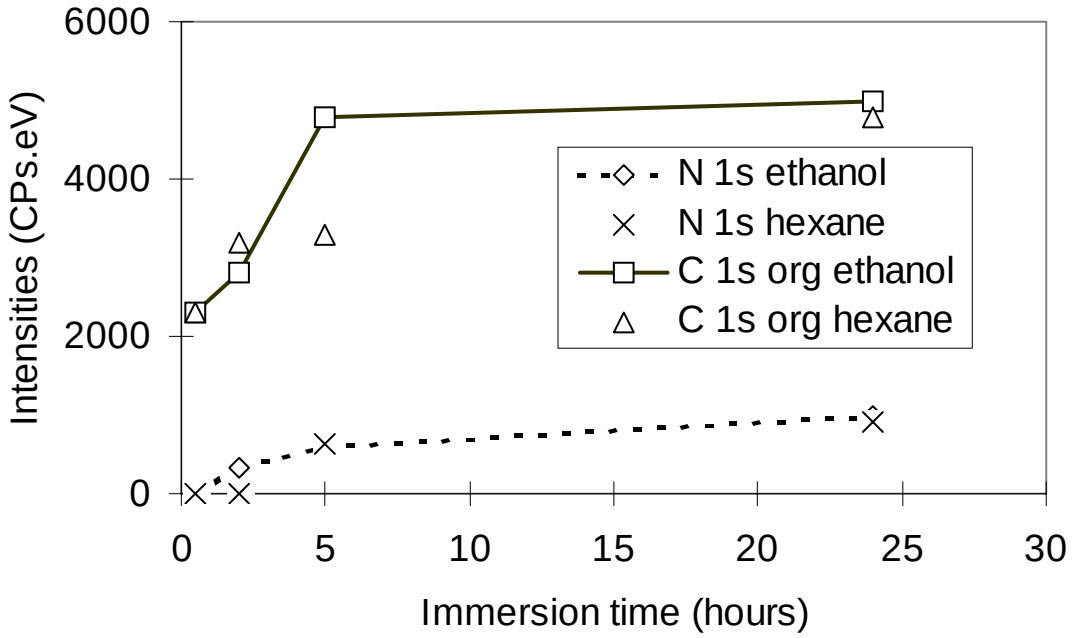


Figure 15: Intensities of N 1s and C $1s^{org}$ signals as a function of immersion time and of cleaning procedures.

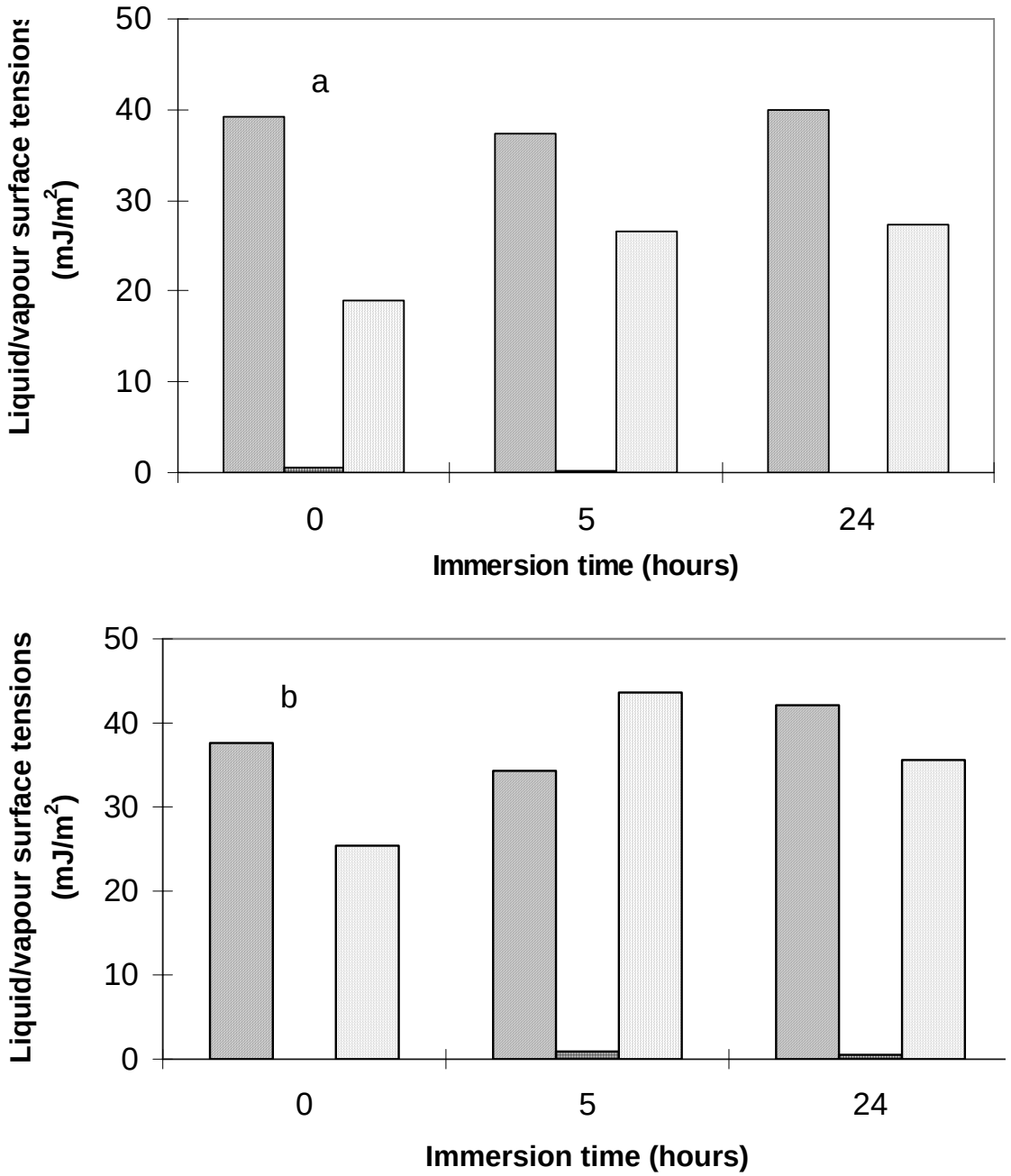


Figure 16: Liquid/vapour surface tensions as a function of immersion time in natural sea water in September 1998 after different cleaning procedures with acetone and ethanol (a) and with hexane (b).

▨ γ^{LW} : van der Waals component, ▥ γ^{+} : electron-acceptor component, ⁞⁞ γ^{-} : electron-donor component