Contributions of oxide film and bacterial metabolism to the ennoblement process: Evidence for a novel mechanism

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Biofilms were grown in laboratory conditions by exposing a range of alloys such as stainless alloys (grade-2 titanium, 6XN, 316L, 904L, Seacure, C276, platinum), aluminium 2S, aluminium 6061, chromium, nickel, molybdenum, copper and cupro-nickel (90:10) to natural pond water. On the basis of photoelectrochemical studies, the oxide films on the above materials were classified as n or p type semiconductors. Alloys having an overlayer of n-type semiconducting oxide film only exhibited a substantial positive shift of corrosion potential. Capacitance measurements were employed to analyse the changes in donor concentration within the oxide film during the ennoblement process. Bacterial and chemical constituents of the biofilm were also analysed. Results of the study strongly suggest that the mechanism of ennoblement is linked to the bacterial removal of excess 'anions' and 'cations' from the oxide film.

BIOFILMS from fresh as well as sea waters usually shift the open circuit potential of stainless steels by several hundred millivolts[1–5]. Johnsen and Bardal[6] suggested that this process of ennoblement is linked to organometallic catalysis of the oxygen reduction reaction. Alternatively, the groups of Dexter hypothesized that the ennoblement is caused by a decrease in pH[2,7,8] at the metal surface by respiring organisms and by the production of peroxide[3]. Maruthamuthu *et al.*[4] proposed that the ennoblement is not an effect of low pH but rather linked to neutral pH conditions. Easwar *et al.*[5] explained that the ennoblement was caused by anodic passivation following neutral pH. Eashwar and Maruthamuthu[9] have also pointed out that siderophores produced by bacteria could be important to ennoblement.

Most recently, Maruthamuthu *et al.*[10] proposed an adsorbed inhibitor theory which emphasized an importance for oxide film in the process of ennoblement. In the present investigation, the contributions of oxide film and bacterial metabolism are examined.

The tests were conducted on grade-2 titanium, stainless steels (316L, 904L, Seacure), super alloys (C-276, 6XN), platinum, aluminium 2S, aluminium 6061, chromium, nickel, molybdenum, copper and cupro-nickel (90:10). The samples were pickled in appropriate acids[11], polished with 400 grade emery, degreased in acetone and rinsed in distilled water prior to all tests. The compositions of the different alloys used are given in Table 1.

For potential measurements, the various alloys were exposed to freshly-sampled pond water in laboratory conditions. Open circuit potentials (OCP) were measured with respect to a saturated calomel electrode (SCE) using a 4 digit high impedance multivoltmeter (HIL 2605). Coupons of 5 cm × 2 cm size were used for potential measurements.

The impact of dissolved oxygen concentration (DO) on potential of titanium was also examined by lowering the dissolved oxygen concentration by the addition of sodium sulphite to the filter-sterilized (0.2 micron) water.

For photopotential measurements, two identical electrodes of each metal/alloy were polished to a mirror finish, degreased with trichloroethylene and lacquered to obtain a geometrical area of 1 cm^2. An all-PVC cell (300 ml capacity) consisting of two compartments, separated by a thin perforated sheet, was so designed that one electrode could always be kept in the dark while the other could be irradiated through a quartz window as and when required. In all the above systems, biofilms were grown on the coupons by renewing the natural pond water every day. Depending upon the direction of the photopotential, the overlaying oxide film was identified as n- or p-type semiconductor.

Capacitance measurements were carried out using a conventional three-electrode electrochemical system with platinum foil as the auxillary electrode and saturated calomel as the reference electrode using PAR electrochemical impedance system. Two 904L coupons exposed to natural and filtered pond water for 40 days were used as the working electrodes.

The nutrient content of the biofilm was analysed at various time intervals after immersion. The biofilm was scrapped using a uniform edged sterilized knife and collected in 100 ml triple distilled water. This sample was used for estimating dissolved nitrite, nitrate, total phosphorus and inorganic phosphates according to Grasshoff *et al.*[12].

Figure 1 shows the open circuit potential (OCP) for 316L stainless steel, chromium, nickel and molybdenum exposed to pond water. It clearly indicates that the OCP of 316L stainless steel alone increases with time to more positive values.

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Table 1. Composition of various stainless steel alloys and grade 2 titanium

Element	904L	AL6XN	Secure	C-276	316L	TiGr2
Al	–	–	0.027	–	–	–
C	0.018	0.019	0.025	<0.02	0.053	0.01
Cr	19.80	20.30	27.36	15.79	17.56	–
Cu	1.52	0.270	0.10	–	–	–
Fe	Bal	Bal	Bal	5.239	Bal	0.07
Mn	1.55	0.52	0.33	0.42	1.60	–
Mo	4.28	6.25	3.53	15.58	2.21	–
Ni	24.10	23.93	2.02	58.93	11.29	–
P	0.019	0.026	0.025	0.006	0.021	–
S	–	0.0003	0.002	<0.001	0.030	–
Si	0.420	0.39	0.41	0.04	0.86	–
Ti	–	–	0.44	–	–	–
Co	0.270	–	0.10	0.22	0.25	–
N	0.057	0.24	0.019	–	0.040	0.005
W	–	–	–	3.76	–	–
V	–	–	–	0.01	–	–
Ti	–	–	–	–	–	Bal
O	–	–	–	–	–	0.111
H	–	–	–	–	–	0.002

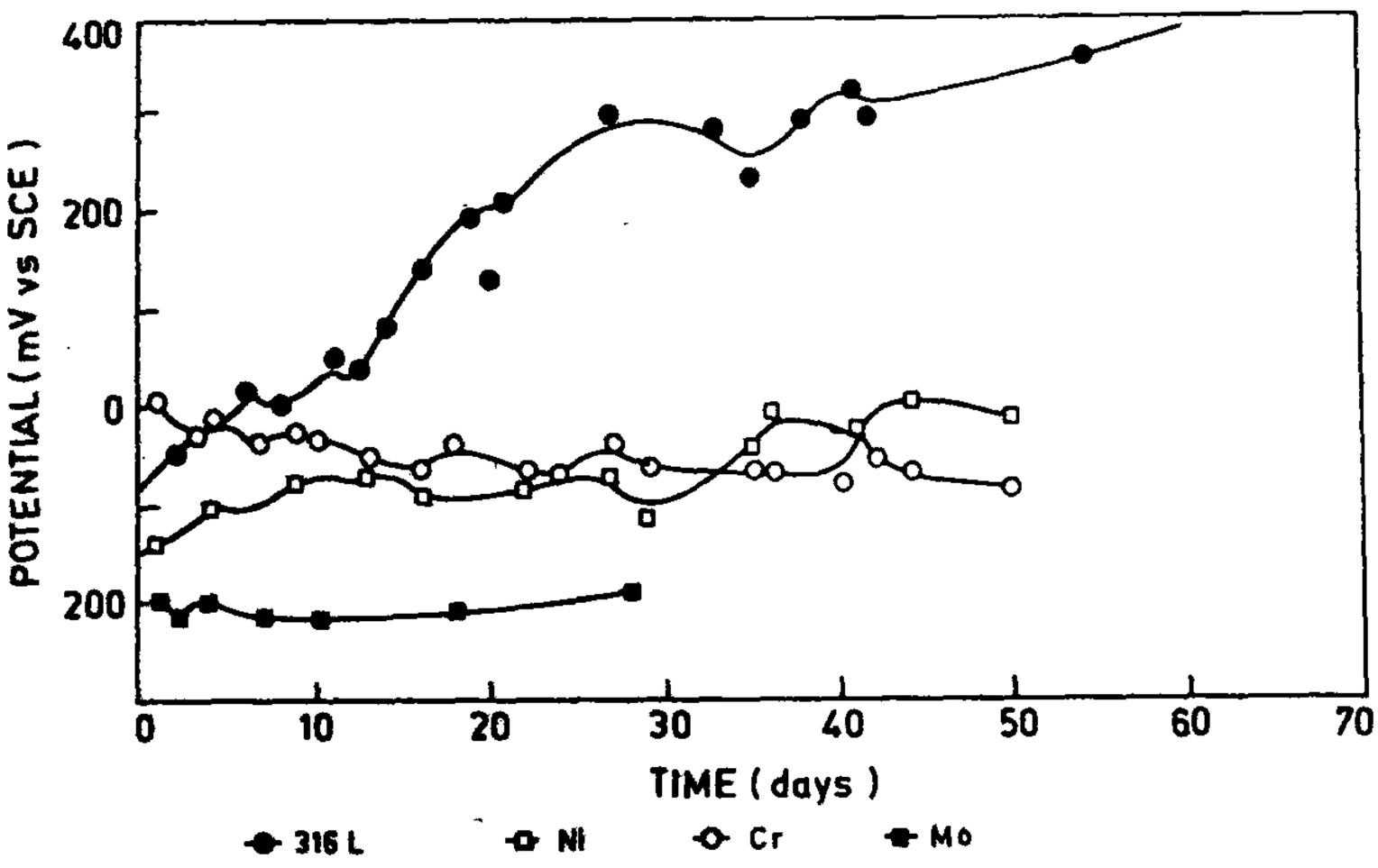


Figure 1. Potentials for 316L SS, nickel, chromium and monel in biotic system.

The OCPs of 6XN, 904L, Seacure and C-276 with time are shown in Figure 2. A large ennoblement is observed for 6XN and the least for C-276.

Table 2 presents the data on the ennoblement range, magnitude of photopotential and semiconducting type of oxide film for various metals/alloys after 32 days of immersion in natural pond water.

The Mott–Schottky ($1/C^2$ vs E) plots for 904L biofilmed electrode and unbiofilmed electrode in pond water are shown in Figure 3. An increase in $1/C^2$ values (i.e., decrease of capacitance) (see ref. 15) is observed on biofilmed specimen as compared to the control specimen. From the capacitance measurement, donor concentrations (at 1000 Hz) as calculated from the slope of the linear region of the curves, are 5.3371×10^{17} for the biofilmed specimen and 1.5929×10^{18} for the bare specimen.

Figure 4 shows the OCP of titanium in filter sterilized deaerated or aerated pond water (pH 8.6 to 9.2) with time. A decrease in dissolved oxygen shifts the potential of titanium to more negative values whereas in deaerated water there is not much fluctuation in OCP.

Figure 5 shows the adsorbed nutrients value of biofilm on titanium. Total phosphorus and nitrate are high in 10 days old biofilm. Estimated nitrite value is low on all days.

Table 2. Data on ennoblement range, amount of photopotential and nature of oxide film for various alloys

Materials	Ennoblement range (mV)	Photopotential	Nature of oxide film
316L SS	300–400	–40	n-type
Chromium	No ennoblement	+10	p-type
Nickel	No ennoblement	+10 – +15	p-type
Molybdenum	No ennoblement	–	Unstable (bluish black)
904L	275–300	–25	n-type
6XN	330–375	–30	n-type
C-276	200	–10 to 13	n-type
Seacure	250	–20	n-type
Titanium	300	–52	n-type
Platinum	450	–50	n-type
Aluminium 2S	No ennoblement	+2	p-type
Aluminium 6061	No ennoblement	+3	p-type
Copper	No ennoblement	+75	p-type
Cupro-nickel	No ennoblement	+60	p-type

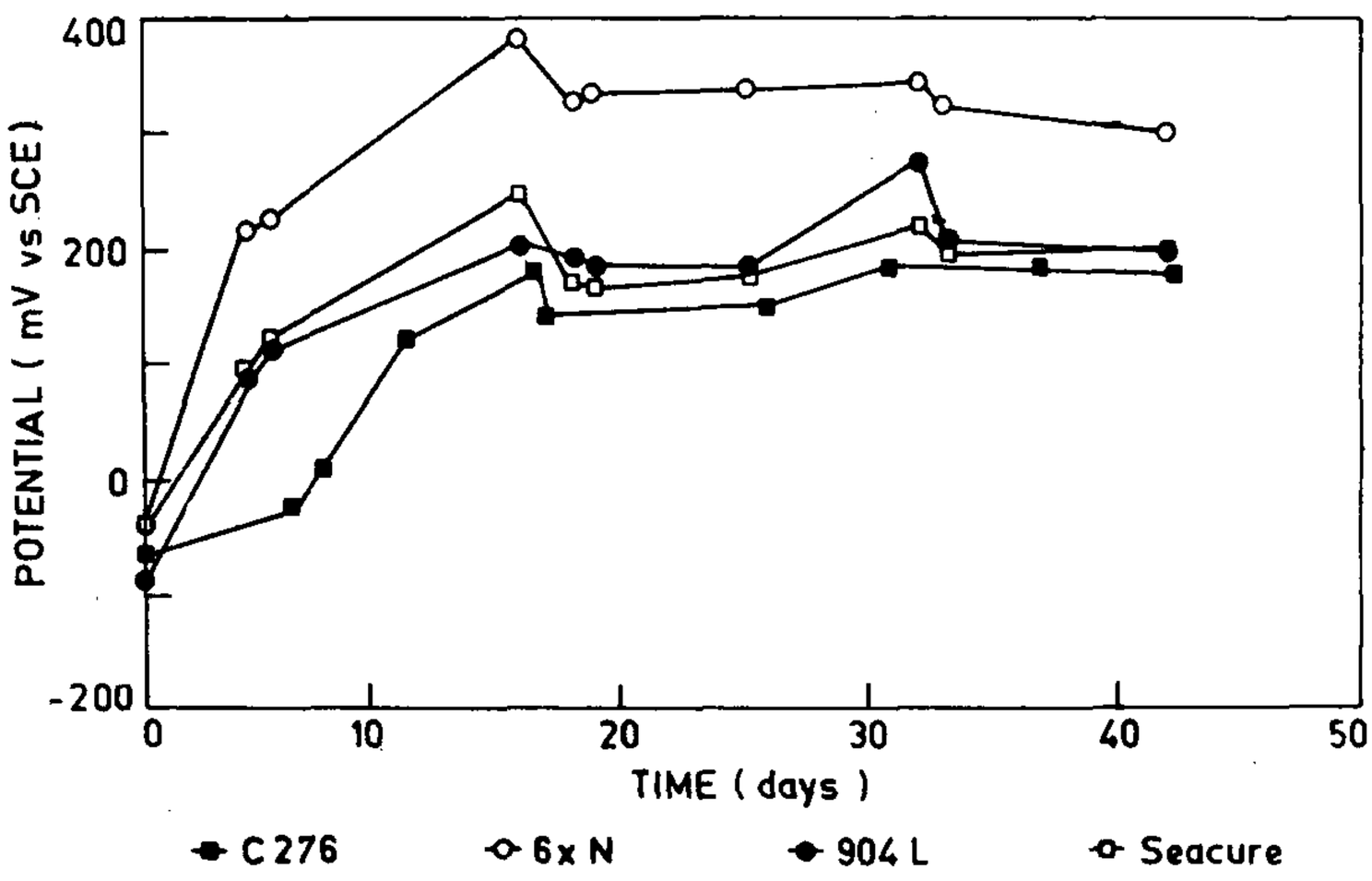


Figure 2. Potentials for stainless alloys of C276, 6XN, 904L and seacure in biotic system.

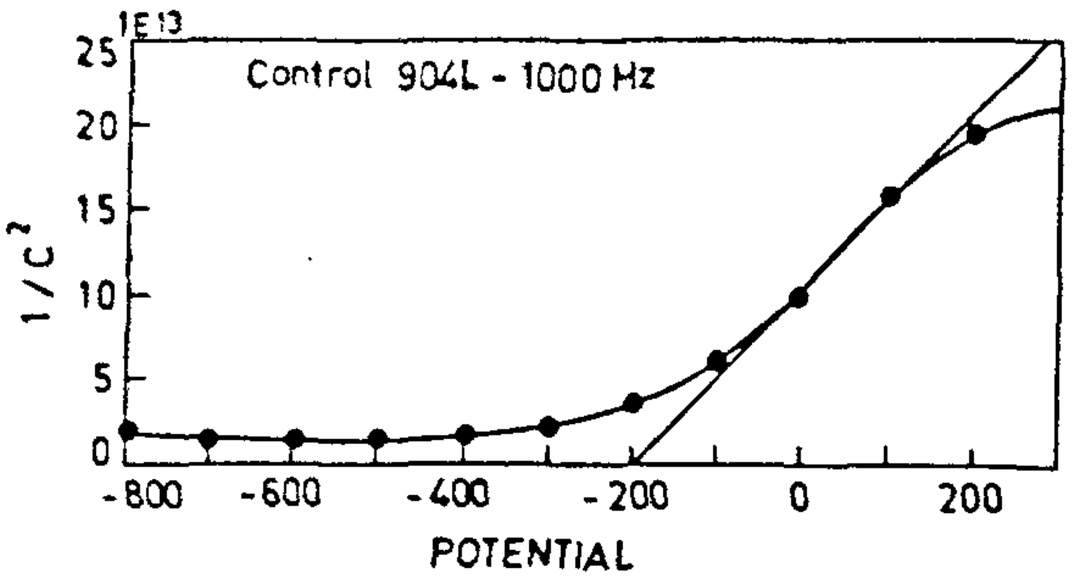
In the present study, the photopotentials have been measured to determine the nature of oxide film on various alloys. The photopotential of a metal oxide can be expressed as

$$V_{ph} = KT/(e \ln (N_A/N_D)), \quad (1)$$

where N_A and N_D are the concentrations of acceptor and donor corresponding to those excessive anions and cations and the stoichiometric composition of oxide film[13,14]. Alloys can be classified as n-type or p-type semiconductors based on the negative or positive shift in potential due to light.

It is seen from Table 2 that a positive shift (ennoblement) in OCP is observed only for metals/alloys having an n-type semiconducting oxide film. Figure 1 explains that while ss 316 shows an ennoblement, the same is not the case for chromium, nickel and molybdenum. This is because chromium and nickel have p-type semiconducting oxide films and molybdenum has an unstable black oxide film. It has to be explained here that even though chromium is present in stainless steel, FeOOH is in outer layer and Cr_2O_3 is in the inner layer[15]. The outer layer therefore acts as an n- type oxide and supports the ennoblement process.

The positive shifts in corrosion potential are large for 6XN, 904L and Seacure but least for C-276 (Figure 2). Results in Table 2 indicate that the semiconducting oxide film on C-276 is an n-type, although the alloy contains high percentage p-type inclusions such as nickel, chromium and molybdenum (Table 1). The high per-



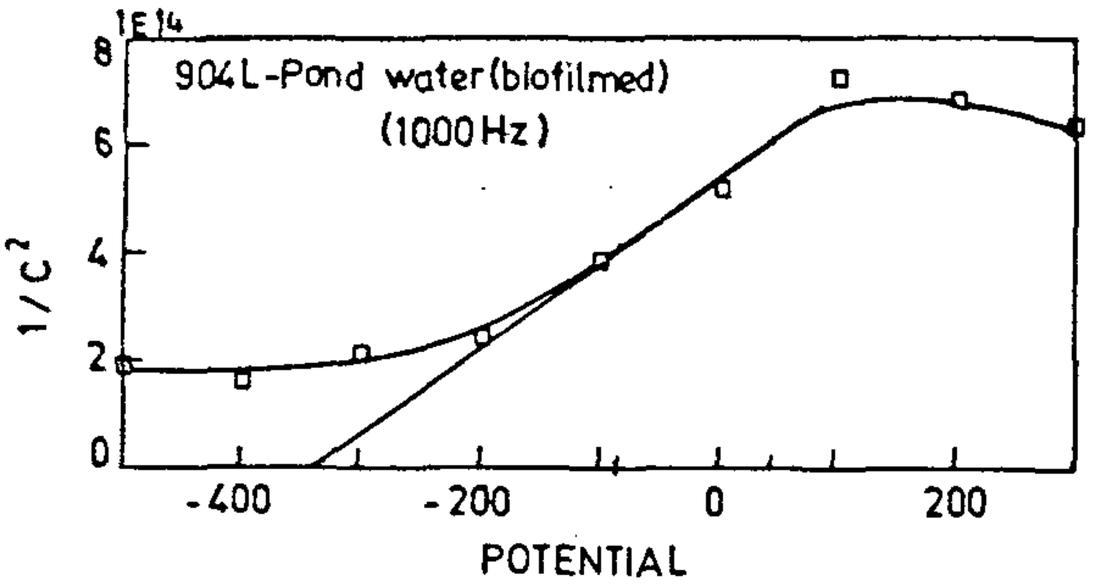


Figure 3. Capacitance vs potential in biotic and abiotic system for 904L at 1000 Hz.

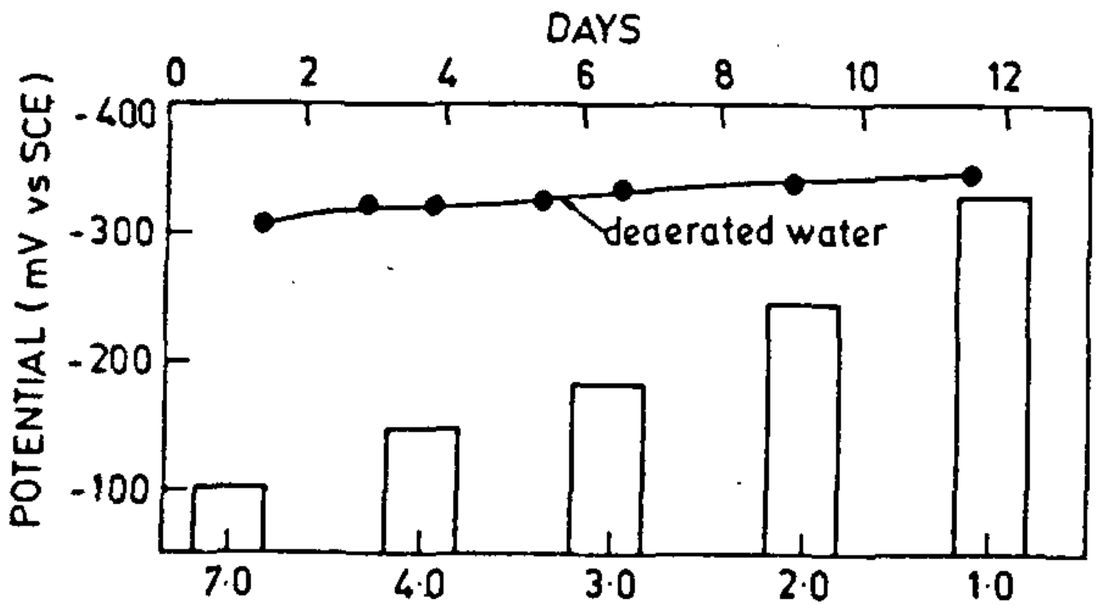


Figure 4. Potential values in various oxygen concentrations.

centage of these inclusions, especially molybdenum[15] might reduce the donor concentration on C-276 alloy and hence be the reason for the least positive shift. But the reason for the high ennoblement range in Delaware waters[8] and Tuticorin (personal observation) for C-276 needs further exploration. The present observation

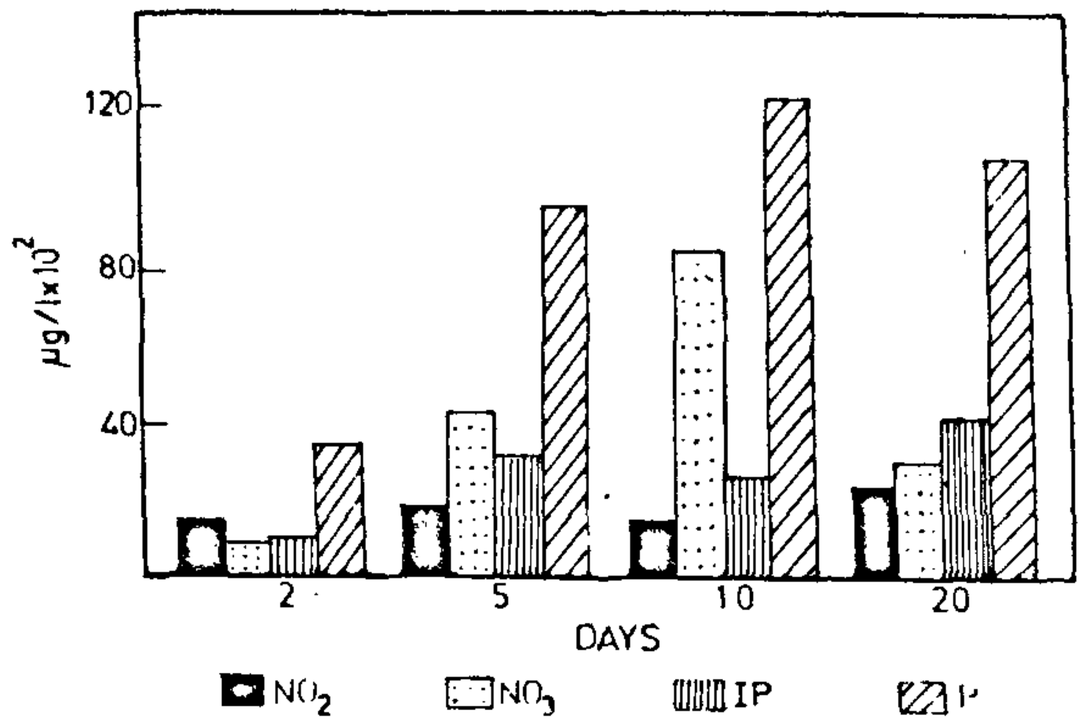


Figure 5. Concentrations of various nutrients in biofilm.

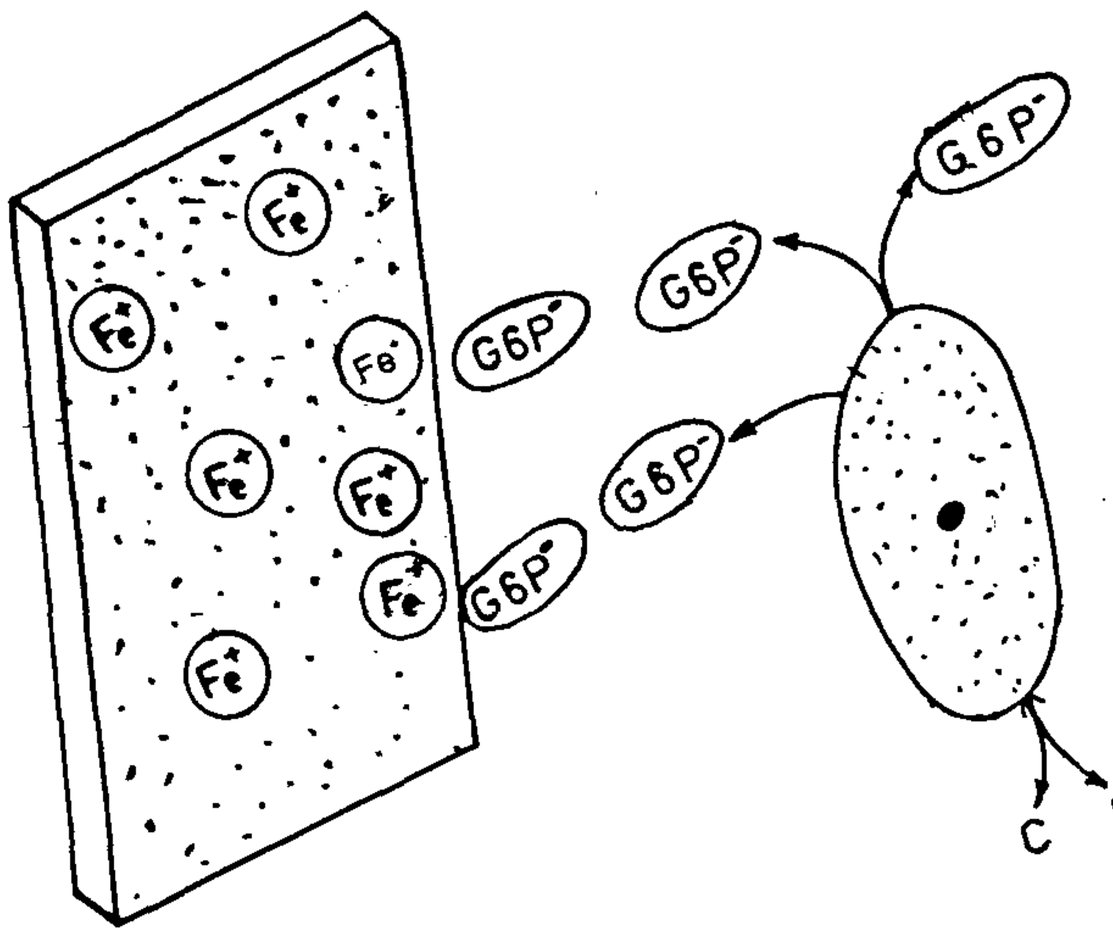


Figure 6. Model for ennoblement mechanism.

suggests that the range of positive shifts for various alloys may depend upon the donor concentration (excess cations) of oxide films. Hence, capacitance measurement has been taken for 904L with and without biofilm.

For a semiconducting/electrolyte interface, the net capacitance C is related to the capacitance of the space charge layer C_{SC} and the capacitance of the Helmholtz layer C_H according to

$$1/C = 1/C_{SC} + 1/C_H. \quad (2)$$

Normally, the capacitance associated with the Helmholtz region is very large compared to that of space charge region and therefore the total capacitance C will correspond to C_{SC}. The $1/C^2$ vs potential plots were analysed using the expression for the space charge capacitance of a semiconductor in the depletion region

$$1/C^2 = 2/(\varepsilon\varepsilon_0 e N_D(V - V_{fb} - KT/q)), \quad (3)$$

where ε is the dielectric constant of the film, N_D the donor concentration, V the applied electrode potential, V_{fb} the flat band potential and the remaining symbols have their usual meaning. According to the equation, the donor concentration can be calculated from the slope of the linear region. The calculation of donor concentration requires the knowledge of the dielectric constant of the films.

In the present study, increased oxide film thickness[10] and decreased donor concentration were observed in presence of a mature biofilm. The capacitance values were also less the biofilmed sample as compared to the control. This result may be explained in terms of the production of anions by bacterial metabolism which could reduce the donor concentration of the oxide film by neutralization with excess cations. The results also suggest that the organic nutrients can act as a semiconductor and enhance the thickness of the oxide film.

According to Chao *et al.*[16], thin passive films contain many lattice defects and as shown by Sato *et al.*[17] for passive iron, the density of these defects acting as electron donors decreases with increasing film thickness because the film tends to take a more stable structure. The lower N_D values obtained in neutral solutions confirm that the amorphicity of the passive films decreases with increasing film thickness. Increasing thickness of the oxide film and anion production by biofilm are a continuous and permanent process which can strengthen the oxide film and make it more stable. This mechanism is similar to the observations made by Irhzo[18] on stainless steel in presence of molybdenum anions. The molybdenum anions MoO_4^{2-} neutralize the positive donors of oxide film, decrease the conductivity and possibly repel the chloride adsorption to increase the pitting resistance of stainless steels.

Figure 4 explains the positive shift in OCP at high oxygen levels. Viera *et al.*[19] also observed the shift in the noble direction in the case of stainless steel when ozone was present in a cooling water system. The positive shifts in chloride solution were also observed by some authors[10,20]. These abiotic positive shifts may be explained as the availability of anions (O^-; Cl^-) may influence the oxide film which may favour the positive shift.

However, Little *et al.*[20] have performed oxygen measurements through microprobe to show that the biofilm substratum interface remained virtually oxygen free. Guezennec[21] clearly showed that an appreciable ennoblement started after 20 days only, when anaerobic bacteria begin to flourish. Further, the sharp influence in corrosion potential was coincident with an increase in the numbers of *Desulphovibrio* and *Desulphatomaculum* species. The potential after a gradual increase up to 50th day, remained unchanged for a further period of 30 days, which was characterized by the domination of these anaerobic species. Recently Eashwar and Maruthamuthu[9] concluded that anaerobic bacterial activity is to be expected in all biofilms. In the present study also, the ennobled specimens of 316L in the OCP of around +390 were maintained up to 6 months in the freshwater. Hence, it suggests that in presence of biofilm, the oxygen and chloride are not needed for ennoblement.

Generally, the oxide growth may occur by cationic movement outwards from the metal/oxygen interface or by anionic movement inwards[22]. Hence, the abiotic and biotic shift may be explained by movement of anions/cations of oxide film.

Figure 5 shows that the biofilm contains high amounts of organic phosphate with nitrates and nitrites. Maloney *et al.*[23] have explained the anion exchange mechanism for both gram-positive and negative cells. The bacteria, for maintenance of a physiological carbon : phosphorus ratio (40:1) during the growth of the cell, brings out the too little carbon and too much phosphorus from the cell in the form of glucose-6-phosphate anion ($G6P^-$) (Figure 6). Bhosle *et al.*[24] have recently identified eight major individual sugars like aralinase, fructose, galactose, glucose, mannose, rhamnose, ribose and xylose in microfouling material. The proposed mechanism is that the anion of organic phosphate combines with excess cations of n-type oxide film leading to both strengthening of oxide film and a positive shift in the corrosion potential. The 'mixed, biologically produced, organic complex' may act as 'anion', strengthen the 'oxygen starved'[21] oxide film for long periods of time. Further study is in progress to present more evidence in support of this novel mechanism.

1. Mollica, A., Trevis, A., Traverso, E., Ventura, G., Scotto, V., Alabiso, G., Marcenaro, G., Montini, V., De Carolis, G. and Dellepiane, R., in Proceedings of the 6th International Congress on Marine Corrosion and Fouling, vol. Marine Corrosion, Athens, Greece, 1984, pp. 269–281.
2. Dexter, S. C. and Lin, S., in Proceedings of the 7th International Congress on Marine Corrosion and Fouling (ed. De Palma, J. R.), Valencia, Spain, 1988.
3. Chandrasekaran, P. and Dexter, S. C., in Proceedings of the 12th International Corrosion Congress NACE International, Houston, 1993, pp. 3696–3707.
4. Maruthamuthu, S., Eashwar, M., Sebastin Raja, S. and Balakrishnan, K., *Biofouling*, 1993, **7**, 257–265.
5. Eashwar, M., Maruthamuthu, S., Sathiyanarayanan, S. and Balakrishnan, K., in Proceedings of the 12th International Corrosion Congress, NACE International, Houston, 1993, pp. 3708–3716.
6. Johnsen, R. and Bardal, E., *Corrosion*, 1985, **41**, 296–302.
7. Dexter, S. C. and Goa, G. Y., *Corrosion '87*, 1987, Paper No. 377, NACE, Houston.
8. Dexter, S. C. and Zhang, H. J., Final Report No. 2939-4, Electric Power Research Institute, University of Delaware, Lewes, Delaware, 1991, pp. 1.1–5.1
9. Eashwar, M. and Maruthamuthu, S., *Biofouling*, 1995, **8**, 203–213.

10. Maruthamuthu, S., Rajagopal, G., Sathiyanarayanan, S., Eashwar, M. and Balakrishnan, K., *Biofouling*, 1995, **8**, 223–232.
11. *Hand Book of Electrochemistry*, Society for Advancement of Electrochemical Science and Technology, Karaikudi (India).
12. Grasshoff, K., Ehrdart, K. and Kremling, M., *Methods of Seawater Analysis*, Verlag Chemie, Vienheim, 1983.
13. Oshe, E. K. and Rozenfeld, I. L., *Electrochimija*, 1968, **4**, 1200–1203.
14. Sathiyanarayanan, S., Manoharan, S. P., Rajagopal, G. and Balakrishnan, K., *Br. Corros. J.*, 1992, **27**, 72–74.
15. Babic, R. and Metikos-Hukovic, M., *J. Electroanal. Chem.*, 1993, **358**, 143–160.
16. Chao, C. Y., Lin, L. F. and Mac Donald, D. D., *J. Electrochem. Soc.*, 1981, **128**, 1187–1194.
17. Sato, N., Azumi, K. and Ohtsuka, T., *J. Electrochem. Soc.*, 1987, **134**, 1352–1357.
18. Irhzo, A., Segui, Y., Bui, N. and Dabosi, F., *Corrosion*, 1986, **42**, 141–147.
19. Viera, M. R., Guiamet, P. S., de Mele, M. F. and Videla, H. A., *Corros. Rev.*, 1993, **11**, 177–185.
20. Little, B. J., Ray, R., Wagner, P., Lewandowski, Z., Lee, W. C., Charaklis, W. G. and Mansfeld, F., *Biofouling*, 1991, **3**, 45–59.
21. Guezennec, J., Scotto, V. and Alabiso, V., in *Microbial Corrosion* (eds Sequiera, C. A. C. and Tiller, A. K.), The Metals Society, London, 1988.
22. Scully, J. C., *The Fundamental of Corrosion*, 2nd Edition, Pergamon Press, New York, 1975.
23. Maloney, P. C., Ambudkar, S. V., Anantharam, V., Sonna, L. A. and Veradhachary, A., *Microbiol. Rev.*, 1990, **54**, 1–17.
24. Bhosle, B. N., Sankaran, P. D. and Wagh, A. B., *Biofouling*, 1990, **2**, 151–162.

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Defective neurulation in frog embryos exposed *to* dilute sea water

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Embryos of *Microhyla ornata* were exposed to dilute sea water from early gastrula stage onwards for a period of 48 h. The process of neurulation was studied in control and treated embryos by using optical and scanning electron microscopy. In the treated embryos, the neural folds formed normally in the initial period of exposure and subsequently approached each other. However, they failed to fuse mid-dorsally in the cephalic region. In the posterior half of the embryos, the neural folds fused to form the neural tube. Irrespective of this, the treated embryos continued differentiation of the brain, as was evident from the development of the eyes. Failure of ectodermal cells to cover the neural cells may be related to the dramatic surface modifications induced due to high concentration of cations like Na^+.

EFFECTS of saline medium on amphibian embryos have been widely studied for various reasons. For example, Ely[1] has studied effects of dilute sea water on embryos and tadpoles of a toad, *Bufo marinus*, and has described the tolerance levels. To find out if acidity of breeding ponds is a limiting factor determining distribution of amphibians in New Jersey, Gosner and Black[2] have investigated effects of altered pH and salinity on the embryonic development of several species of frogs. With a view to understanding the ecological relationship of amphibians to brackish water, Ruibal[3] has studied effects of salinity on embryos of *Rana pipiens*. Salthe[4], who was interested in finding out the mechanism of increase in the volume of perivitelline space during early amphibian development, has also reported effects of low pH, various cations and anions on the embryos of *R. pipiens*.

In recent years, Beebee[5] has studied salt tolerance of the embryos of natterjack toad (*Bufo calamita*) because breeding ponds of these toads are subjected to salt spray and tidal inundation in some coastal areas of Britain. Desiccation of breeding ponds due to irregular rainfall and possibility of tidal inundation of ponds in coastal areas prompted Padhye and Ghate[6] to investigate salt tolerance of the embryos of *Microhyla ornata*.

While studying the ecology of brackish water population of *R. pipiens* from California, Ruibal[3] has made an interesting observation regarding neurulation of the embryos exposed to near-lethal concentration of sea water – it has been mentioned that at salinities above 5‰ the surviving embryos displayed anteriorly open neural groove. Similar effects have latter been photographically documented along with brief histology of defective neural tube[6].

In this paper we provide additional evidence in the form of histological and scanning electron microscopic (SEM) analyses of defective neurulation in *M. ornata* embryos exposed to dilute sea water. The interesting facts emerging out of this work are: (1) the mechanism of neural tube closure may be different along the anteroposterior axis of the neural tube, (2) exposure to saline medium leads to collapsing of elevating neural folds as well as detachment of nonneural and neural ec-

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