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Impact of biological factors on the ennoblement of stainless steel in Baltic seawater

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Open circuit potentials of stainless steels increased when immersed in the Baltic Sea. The ennoblement potential was +200 mV_{sce} in 40 to 50 days when sea water temperature was below 5°C and +300–400 mV_{sce} within <40 days at around 10°C. Ennoblement occurred in a laboratory ecosystem at 23°C in 20 to 30 days, and at 26°C in <20 days, but no ennoblement occurred at ≥32°C within 40 days. By the time the ennoblement was complete, compact microcolonies covered 1–10% of the steel surface. Nutrient enrichment of Baltic Sea water by twofold above the natural levels increased microbial growth but attenuated open circuit potential increase of the stainless steels. Exposure of the ennobled stainless steels to similar levels of nutrients did not reverse the already developed open circuit potentials. Attenuation of the ennobling response of the stainless steels by increases of temperature and eutrophication suggests a role for microorganisms which is crucial for the electrochemical behaviour of steels in brackish Baltic Sea water. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 410–420.

Keywords: biofilm; stainless steel; ennoblement; Baltic Sea; season; laboratory ecosystem

Introduction

The development of ennoblement in stainless steel is believed to precede corrosion of the steel. Conditions involved have been studied intensively in oceans and in the Mediterranean [6,12,15]. It is not known if similar processes occur in low-salt environments. The Baltic Sea is the world's largest brackish water basin. It is heavily used as industrial cooling water, e.g. in power plants using fossil and nuclear (14 GW on the Baltic Sea shoreline [5]) fuels and intensively used by sea traffic. Similar low-salt environments are found on all continents in estuarine situations. In earlier work we demonstrated that ennoblement of stainless steels occurred in brackish Baltic Sea water in a laboratory ecosystem [9,14].

In this study we investigated biotic and abiotic factors affecting the ennoblement in different stainless steels exposed to Baltic Sea water in the field and in a laboratory ecosystem.

Materials and methods

Stainless steels coupons

Stainless steel UNS S31600 (%, w/w: 0.03 C, 0.56 Si, 1.51 Mn, 0.04 S, 16.9 Cr, 10.7 Ni, 2.6 Mo), UNS S31254 (%, w/w: 0.01 C, 0.42 Si, 0.40 Mn, 0.002 S, 20.1 Cr, 17.2 Ni, 6.1 Mo, 0.67 Cu, 0.2 N), UNS S30400 (%, w/w: 0.04 C, 0.46 Si, 1.48 Mn, 0.005 S, 18.4 Cr, 8.6 Ni, 0.1 Mo, 0.2 Cu) and UNS N08904 (%, w/w: 0.02 C, 0.53 Si, 1.48 Mn, 0.002 S, 19.5 Cr, 25.0 Ni, 4.5 Mo, 1.4 Cu) were used as discs of 25 mm (field experiments) or 13–15 mm (laboratory experiments) diameter and as coupons of 25 mm×75 mm with a thickness of 2 mm (for epifluorescence in field experiments).

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The steel surface was used as delivered (cold-rolled, annealed, pickled and finishing rolled for field experiments) or polished to 600 grit (disks for laboratory experiments).

Experimental set-up in the field and in the laboratory

Test coupons were mounted in specimen holders for exposure in the Baltic Sea (depth: 15 m) in the Helsinki coastal area in 1995–1997 and in a laboratory ecosystem [8,14]. Natural brackish Baltic Sea water from the vicinity of the field exposure site was used as feed for the laboratory ecosystem. Hydraulic retention times in the light (1850 lux, 12 h day^{-1}, 100 l) and in the dark compartments (25 l) of the laboratory ecosystem were adjusted to balance the mineralization of organic carbon (dark reaction) by the primary production (light reaction) so that the concentration of dissolved organic carbon was maintained at the natural level, 4 to 7 mg of C l^{-1}. The laboratory ecosystem was fed from an 800-l storage container (filled with new sea water every 14 days) which was aerated for 30 min/day. Fertilization of the feed was done (when indicated) with ammonium chloride and potassium phosphate.

Electrochemical assays

Open circuit potentials were measured at the field test site using a digital voltmeter (Fluke 77) and a silver/silver chloride (Ag/AgCl, 3 M KCl) or a saturated calomel (SCE) reference electrode. The redox potential and the pH of the Baltic Sea water were determined within 2 h of taking samples. Water was maintained at its sampling temperature until measured. In the laboratory ecosystem a modified Robbins device [7] was used. Open circuit potentials were measured using a potentiostat (Wenking LB81M, Germany, or Gamry Instruments PC3, Warminster, PA, USA) with a saturated calomel reference electrode.

Microscopy

For epifluorescence microscopy the steel coupons were stained with aqueous acridine orange (0.01%, Difco, Detroit, MI, USA) and rinsed with sterile water. Stainings with CTC (5-cyano-2,3-ditolyl tetrazolium chloride, Polysciences, Warrington, PA, USA) and with DAPI (4′,6-diamidino-2-phenylindole, Sigma, St. Louis, MO, USA) were performed according to Steward *et al.* [17] and Yu and McFeters [19]. The stained coupons were air dried and stored at +4°C. Stained surfaces were examined with an Olympus BHT epifluorescence microscope (Japan) using B-filter (excitation filter 370-490) and photographed through a 100× oil-immersion objective.

Scanning electron microscopy (SEM, JMS-840, Jeol, Tokyo, Japan) was performed as described by Väisänen *et al.* [18]. For SEM (Jeol 6400, Jeol, Akishima, Japan) for the inspection of corrosion damage, the coupons of stainless steel were cleaned by acid pickling (8.4% aqueous nitric acid at 20°C for 2 min). Confocal laser scanning microscopy (CLSM) was performed with a Zeiss LSM Invert, 40× oil immersion objective and a Bio-Rad MRC-600 inverted, 40× water immersion objective. The steel coupons were rinsed with filtered Baltic Sea water and stored in sterile filtered Baltic Sea water (no fixation) until stained and examined. The coupons were stained using aqueous solutions of syto 16 (20 μM, 20 min, Molecular Probes, Eugene, OR) and tetramethylrhodamine-tagged concanavaline A (20 μg ml^{-1}, 20 min) in the given order or 0.03% acridine orange (Sigma) in 0.2 M acetic acid buffer pH 4 for 5 min. Microscopic examination was performed within 6 h of staining.

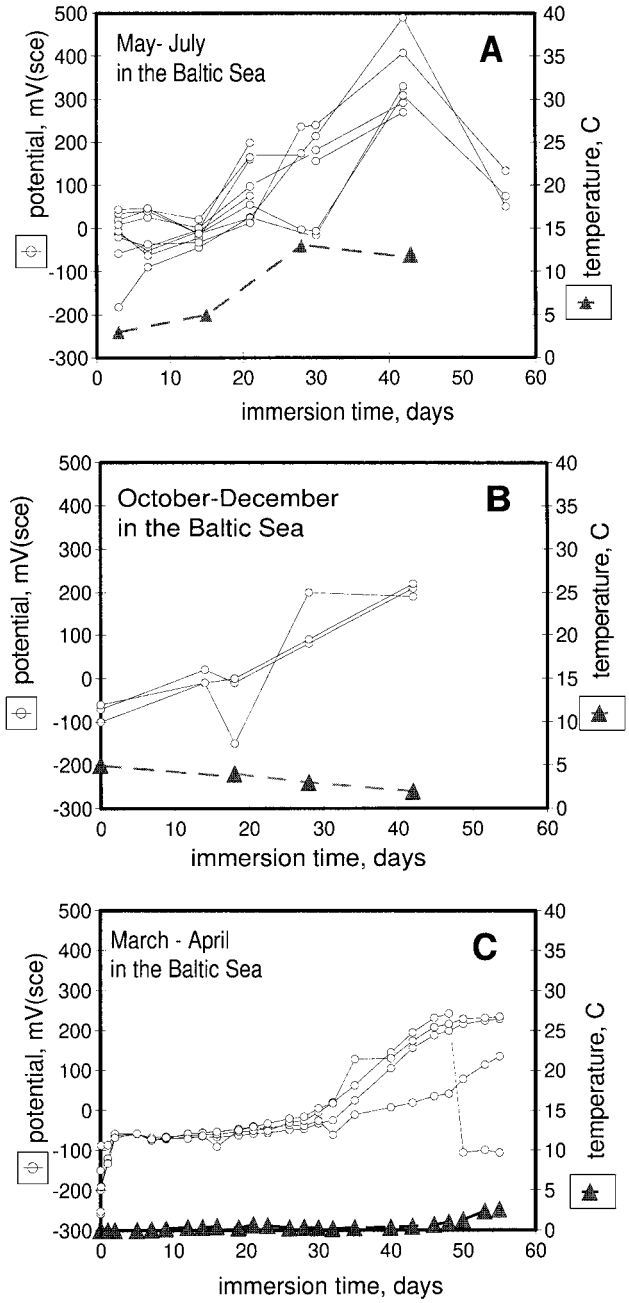

Figure 1 Development of ennoblement in stainless steel coupons (UNS S31600) immersed in the Baltic Sea in different seasons. Temperature increased from 3°C (May) to 12°C (July) (A), decreased from 5°C to 2°C during October to December (B) and was close to 0°C during March to April (C). The lines indicate the electrochemical behaviour of individual coupons. The number of replicate coupons was nine in panel A, three in panel B and four in panel C.

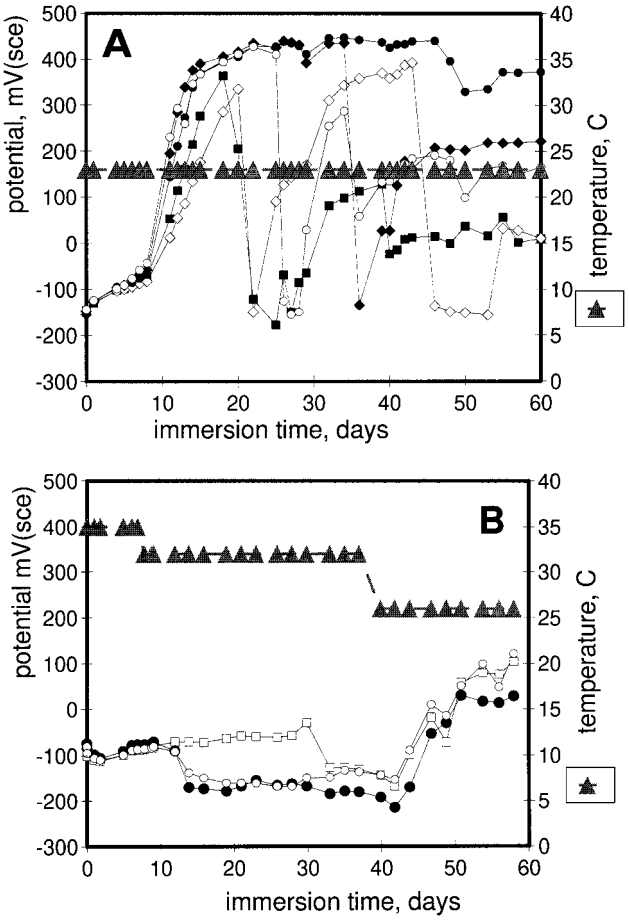

Figure 2 Development of ennoblement in stainless steel coupons (UNS S31600) immersed in Baltic Sea water in the laboratory at 23 (A), 35, 32 to 26°C (B). The lines indicate the electrochemical behaviour of individual steel coupons.

Analytical protocols

For the measurement of biofilm ATP the metal coupons were rinsed with Tris (0.1 M)–EDTA (2 mM) pH 7.8 adjusted with acetic acid, immediately placed in boiling Tris–EDTA buffer (2 to 5 ml per coupon) for 5 min and rapidly cooled in an icebath. One hundred microliters of the cooled sample, 100 μl of ATP monitoring reagent (luciferin-luciferase of firefly; Bio-Orbit, Turku, Finland) and 100 μl of ATP standard solution (internal standard; 10^{-7} M in sterile distilled water; Bio-Orbit, Turku, Finland) were added to the sample in the order given. A luminometer (Bio-Orbit, Turku, Finland) reading was taken after the addition of each reagent. ATP content was calculated on the basis of the response obtained from the internal standard.

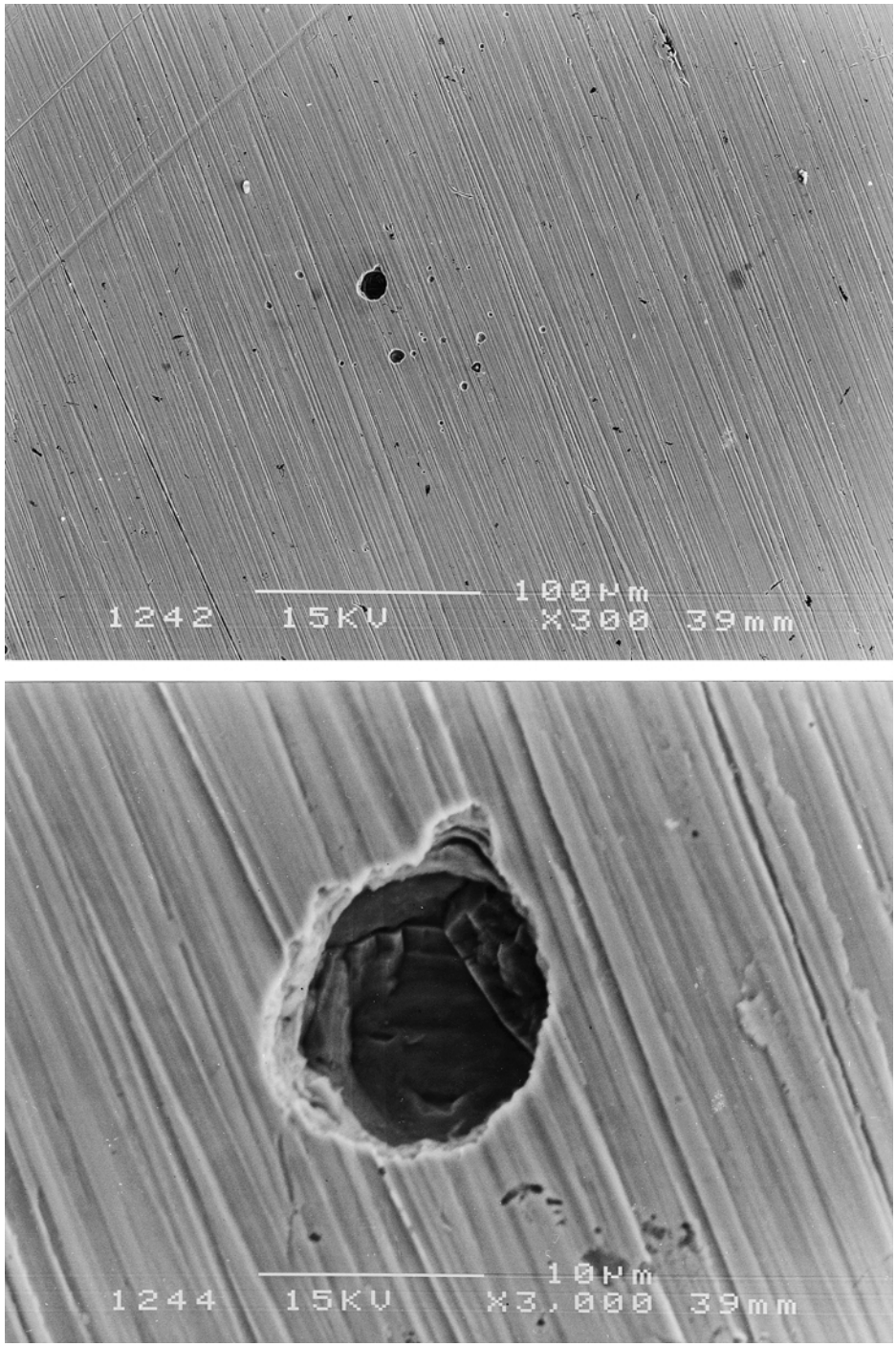

Figure 3 SEM micrographs of a typical small pit nucleased on the surface of stainless steel (UNS S31600) after 56 days of immersion in Baltic Sea water at 23°C in the laboratory ecosystem (Figure 2A). The lower panel represents a higher magnification of the steel coupon shown in the upper panel.

Table 1 Conditions for the ennoblement of stainless steels and composition of the Baltic Sea water used in the laboratory ecosystem. The pH of Baltic Sea water was 7.3 to 7.9

Laboratory ecosystem experiment		Steel parameters			Feed parameters								
		Type of steel (UNS) and positioning of coupons	E_{OC} max mV (n)*	Ennoblement day	Flow (mm s^{-1})	TOC (mg Cl^{-1})	Total P ($\mu g\ l^{-1}$)	Total N ($\mu g\ l^{-1}$)	Cl^- (mg l^{-1})	Mn ($\mu g\ l^{-1}$)	Al ($\mu g\ l^{-1}$)	Fe ($\mu g\ l^{-1}$)	SO_4^{2-} (mg l^{-1})
L3	May to August	N08904 S30400 horizontal	+180 (6) +140 (3)	(>67) (>67)	<1	5.9 SD 0.3 *n* 4	31 SD 27 *n* 4	492 SD 30 *n* 4	3050 SD 900 *n* 4	25 SD 6 *n* 4	ND	83 SD 29 *n* 4	440 SD 74 *n* 4
L4	August to September	S31254 horizontal	+100 (8) +150... +200 (4)	(>67)	<1	3.7 SD 2.6 *n* 3	32 SD 12 *n* 3	532 SD 65 *n* 3	3166 SD 288 *n* 3	23 SD 6 *n* 3	43 SD 42 *n* 3	73 SD 32 *n* 3	447 SD 21 *n* 3
L6	March to May	S31600 S30400 S31254 vertical	+360... +400 (6) +350... +380 (3) +370... +400 (3)	19–21 21–22 19–21	5	4.1 SD 1.3 *n* 5	26 SD 6 *n* 5	522 SD 158 *n* 4	2725 SD 96 *n* 4	23 SD 6 *n* 4	163 SD 85 *n* 4	118 SD 24 *n* 4	388 SD 22 *n* 4
L8	July to September	S31600 S31254 S30400 vertical	+350... +430 (6) +420... +430 (3) +270... +420 (3)	15–21 21 21–25	10–35	4.5 SD 0.8 *n* 5	29 SD 5 *n* 7	376 SD 99 *n* 7	2929 SD 76 *n* 7	53 SD 15 *n* 7	124 SD 38 *n* 7	148 SD 67 *n* 7	518 SD 7 *n* 7
L10	March to May	S31600 S31254 vertical	+160... +333 (6) +350 (3)	18–29 21–29	10–35	6.6 SD 1.4 *n* 5	33 SD 5 *n* 6	766 SD 178 *n* 6	2617 SD 232 *n* 6	16 SD 9 *n* 6	199 SD 147 *n* 6	193 SD 102 *n* 6	387 SD 31 *n* 6

*Number of coupons exposed.

The biofilm dry weight (2 h, 105°C) was calculated as the difference of weight of the dried coupon before and after the biofilm was mechanically removed from the coupon.

The viable count of bacteria was estimated by wiping the biofilm on the test coupons with an alginate swab. The swab was immersed in sterile 0.9% NaCl, dilution plated in maximal recovery diluent (LabM, Bury, UK) on plate count agar (Difco) and grown for 5 days at 25°C.

Total organic carbon (TOC) was measured according to ISO standard 8245 [3]. Nitrite, nitrate, total nitrogen and total phosphorus were measured according to APHA protocols [2].

All chemicals were of analytical quality unless otherwise stated.

Results

Ennoblement of stainless steels in the Baltic Sea water

Effect of temperature on ennoblement: Stainless steel coupons (UNS S31600) were immersed in the Baltic Sea in the early summer (from May to July), in the autumn (from October to December) and in the winter (from March to April). Open circuit potentials (ennoblement) of the immersed stainless steels increased with time in all seasons (Figure 1). The increase was complete in <40 days in the summer (3°C to 12°C, Figure 1A) and in 40 to 50 days in the cold season (0° to 5°C, Figure 1B, C). The ennoblement potential levelled off at +200 mV_{sce} in water near 0°C and at +300 to +400 mV_{sce} in water around 10°C. The lag period before the potential started to increase, was longer at 0°C (30 days) than at around 10°C (15 days) (Figure 1).

The impact of temperature on the development of open circuit potential of stainless steel was further studied in a laboratory microcosm. The steels immersed in Baltic Sea water in the laboratory at 23±1°C gained ca. +400 mV in potential within 20 days (Figure 2A). Coupons immersed in Baltic Sea water maintained at 32°C or at 36°C showed no ennoblement within 38 days (Figure 2B). When the temperature was decreased to 26°C the open circuit potential increased within 20 days (Figure 2B). The final open circuit potential was lower, ≤200 mV, in steels exposed at 26°C (Figure 2B) than in those exposed at 23°C (>400 mV, Figure 2A). The fluctuations of the potentials seen in Figure 2A, after ennoblement may indicate the initiation of pitting and/or crevice corrosion. After the test period small (10–20 μm) pits and slight crevice corrosion was seen on these coupons with SEM (Figure 3).

The composition of the brackish Baltic Sea water and the development of ennobling potentials in the laboratory ecosystem are summarized in Table 1. At 23±1°C a voltage increase to the level of +300 to +430 mV_{sce} was observed in the laboratory ecosystem experiments L6 and L8 but not in L3 and L4. The data from the five independent series of laboratory experiments fed for 40 to 67 days each with water collected from the Baltic Sea in different seasons were very similar indicating that natural seasonal fluctuations (Table 1) in the composition of the brackish Baltic Sea water did not explain differences in the ennoblement behavior (Tables 1 and 2). Summarising the data in Figures 1 and 2 and Table 1, the ennoblement of stainless steel coupons occurred at temperatures between 0°C and 26°C, but not at ≥32°C in <50 days.

Properties of biofilms grown on stainless steel coupons immersed in the Baltic Sea (0 to 12°C) at a depth of 15 m or in the laboratory ecosystem (23±1°C) are summarized in Table 2. The biofilms formed on steel coupons ennobled in the field (at sea) had a higher dry weight (0.9 to 5.1 mg cm^{-2}) than those grown in the laboratory ecosystem fed by the same water (0.3 to 0.6 mg cm^{-2}). There was no meaningful difference in the biofilm viable counts (10^4 to 10^5 cfu cm^{-2}) or the amount of biomass between the biofilms grown on ennobling and non-ennobling stainless steel coupons in the laboratory or in the field experiments (Table 2). The observed biofilm ATP contents (≈5 pmol cm^{-2}) indicate that if a threshold level exists for the ennoblement to occur, it is low. These results indicate that ennoblement of stainless steel coupons may depend more on the quality of biofilm than on the quantity of biofilm biomass.

Table 2 Properties of biofilms grown on stainless steel in Baltic Sea water in a laboratory ecosystem and in the field. The laboratory ecosystem (150 l) was fed by Baltic Sea water at the rate of 0.05 to 0.06 (vol/vol day^{-1}) and the temperature was 23±1°C

Experiment		Flow (mm s^{-1})	Metal (UNS) and position	Exposure days	Ennoblement	Biofilm dry weight (mg cm^{-2})	Biofilm ATP content (pmol cm^{-2})	Biofilm viable count (log cfu cm^{-2})
Code	Months							
Laboratory experiments								
L4	August to September	1	S31254 horizontal	21	no*	0.35 (±0.1)	5.9 (±0.27)	4.2 (±0.02)
L6	March to May	5	S31600 vertical	21	yes*	ND	11.2 (±0.09)	4.9 (±0.03)
L7	June to July	5	S31600 vertical	21	no	0.29 (±0.1)	2.1 (±1.74)	ND
L8	July to September	10–35	S31600 vertical	21	yes*	ND	19.2 (±0.05)	ND
Field experiments								
F1	May to December	ND	S31600	156	yes	5.1	5	3.6 (±0.11)
F2	May to December	ND	S31600	84	yes	0.93 (±0.43)	115 (±5)	5.5 (±0.38)

*See for details Table 1.

The effect of nutrient enrichment on the ennoblement

The Baltic Sea water used as the feed in the laboratory was brackish, oligotrophic water with 20 to 40 μg of total P l^{-1} and 300 to 800 μg of total N l^{-1} (Table 1). The feed of the laboratory ecosystem was spiked with salts of nitrogen and phosphorus calculated to increase twofold the levels of N and P. No ennoblement of the stainless steel (UNS S30400 and S31600) disks immersed in the enriched feed was observed within 70 days. The Helsinki City municipal waste water treatment plant (600,000 inhabitant units) had a technical problem, resulting in an increased discharge of waste water in the coastal area from which water was taken to the laboratory ecosystem. As a result, the laboratory ecosystem feed contained twice as much total N but no more total P than the usual feed (Table 1). During the follow-up period of 67 days only four out of 12 steel disks (UNS S30400 1/3, UNS S31600 2/6 and UNS S31254 1/3) ennobled.

In order to see whether the observed attenuation of ennobling of stainless steels by nutrients was a reversible phenomenon, we fertilized the feed of the laboratory ecosystem with salts of nitrogen, phosphorus and a carbon source (13 mg of N_{tot} l^{-1} and 0.14 mg of P_{tot} l^{-1}, 16 mg glucose-C l^{-1}, once a week) after the steels (UNS S31600 and UNS S31254) had ennobled. No decrease of the open circuit potentials was observed of any of the coupons that had already ennobled 20 days earlier or with coupons that had just reached ennoblement ($+220\,mV_{sce}$) during the follow-up period of 20 days.

Enrichment with nutrients may thus have attenuated ennoblement but once ennoblement had occurred, it was not reversed by introduction of nutrients.

Microscopic structure of biofilms on ennobling stainless steels in Baltic Sea water in the field and in the laboratory

CLSM images and epifluorescence microscope views (Figure 4) of stainless steel coupons immersed in the Baltic Sea during the cold season showed that the pre-ennoblement biofilm (14 days immersed, Figure 4A) was thin, with $\geq 10^2$ bacterial cells attached to 1000 μm^2 of steel surface. There was no sign of concanavaline A (glucose and mannose specific lectin) reactive substances. After ennoblement increased close to the plateau value of $+250\,mV_{sce}$, after 55 days of immersion (Figure 4B) one to a few microbial communities per 1000 μm^2 developed which were stainable with rhodamine-tagged concanavalin A. The sizes of microbial patches attached to the stainless steel surfaces ranged in diameter from 10 μm to 50 μm. Acridine orange staining also indicated that there were ≤ 10 microbial communities per 1000 μm^2 of steel surface, separated by areas with no, or only single, cells (Figure 4C).

The microscopic structure of ennobling biofilms grown on stainless steel in the Baltic Sea from spring to summer season is shown in Figure 5. After immersion for 28 days, before the

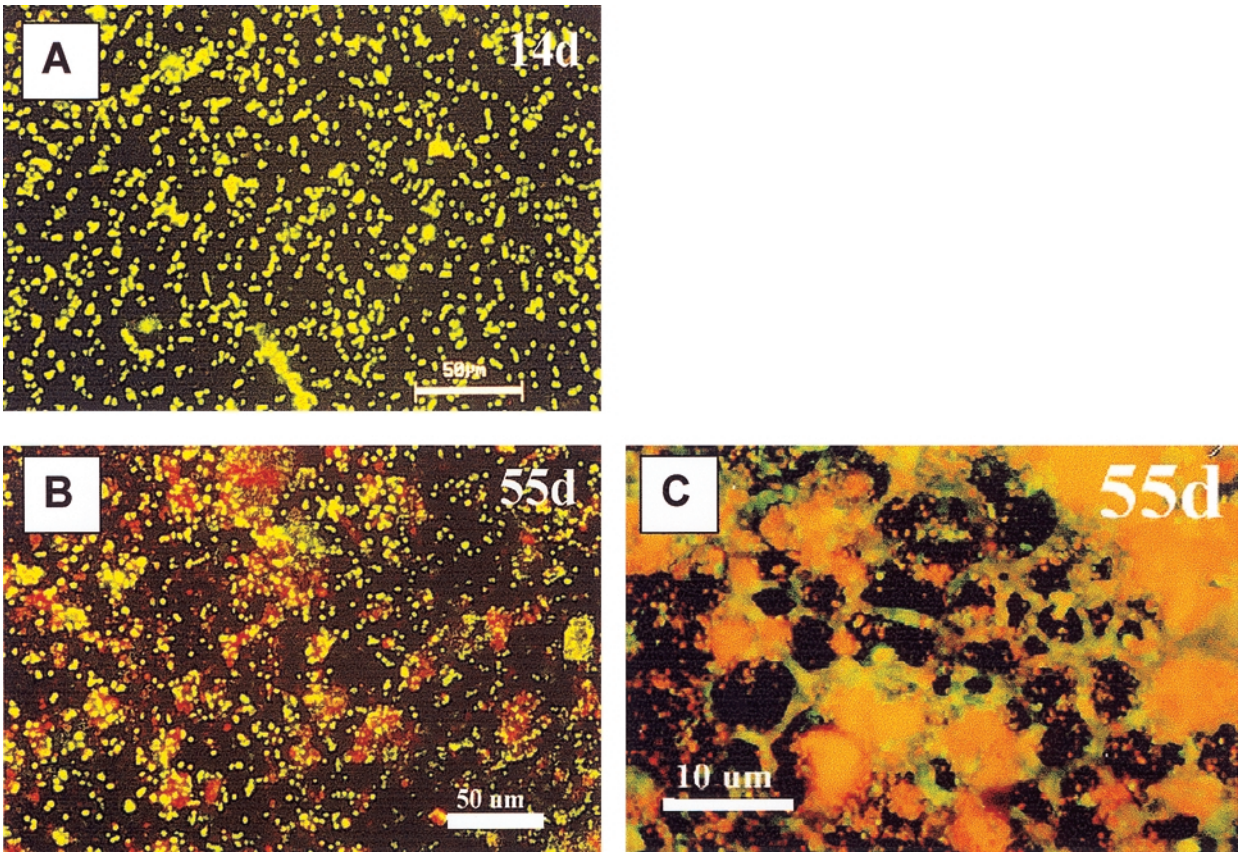

Figure 4 CLSM (A and B) and epifluorescence (C) micrographs of biofilm on stainless steel (UNS S31600) ennobled in the Baltic Sea during the winter season. The figure shows images (average of eight individual optical sections of 1 μm each) of biofilms on stainless steel surface after 14 days (A) and 55 days (B, C) of immersion in the Baltic Sea, from March 1st onwards. The biofilms were stained with rhodamine-tagged concanavalin A (red, specific for glucosyl and mannosyl residues) and syto16 (green, DNA stain, A and B) or acridine orange (C).

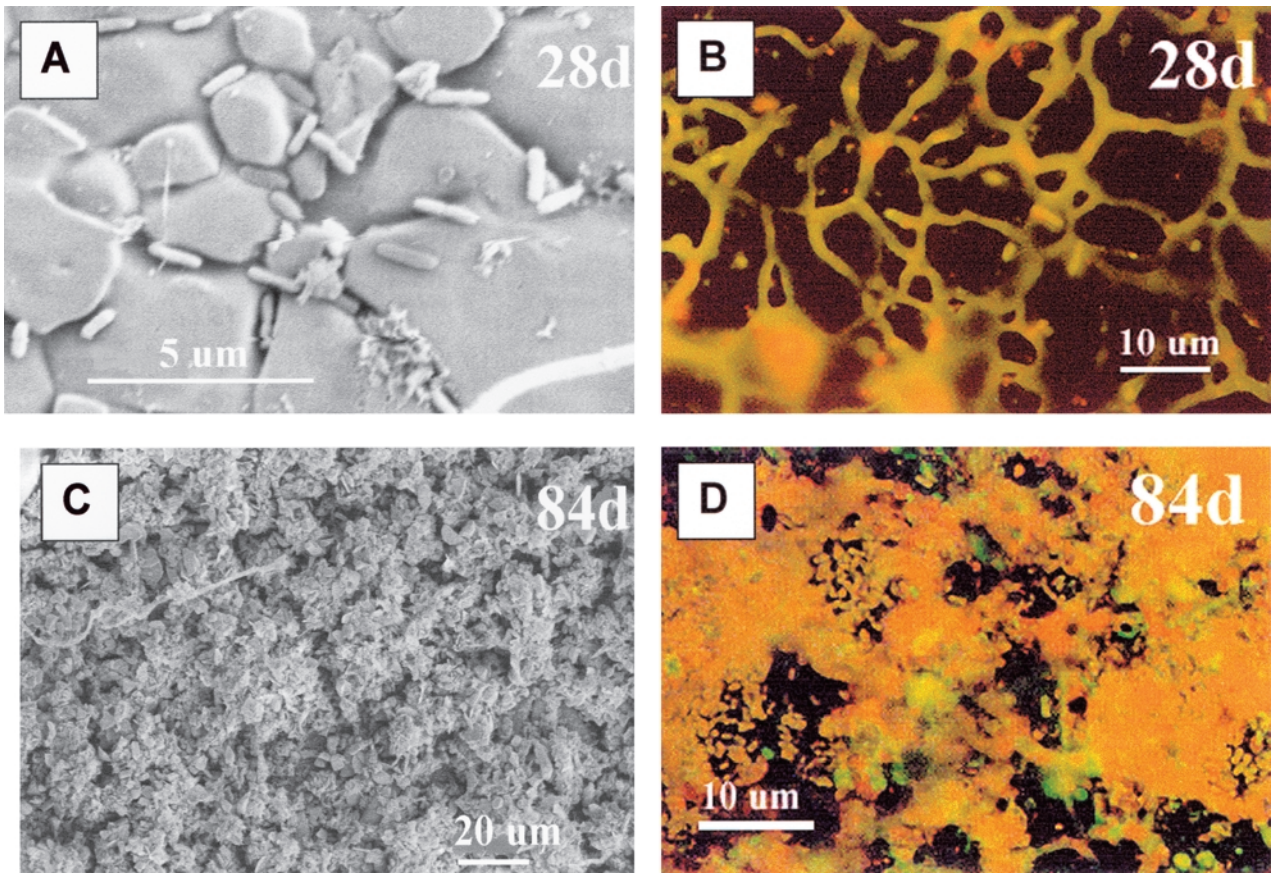

Figure 5 Micrographs of biofilms on stainless steel (UNS S31600) ennobled in the Baltic Sea in the warm season. Ennoblement potential (300 to 450 mV_{sce}, Figure 1A) was reached after 28 days of exposure. The figure shows biofilms on surfaces of stainless steel after 28 days (A, B) and 84 days (C, D) of immersion in the Baltic Sea from May 16th onwards (temperature 3°C to 12°C, Figure 1A) viewed by scanning electron microscope (A and C) and epifluorescence microscope, stained with acridine orange (B and D).

ennoblement was complete, the biofilms on steel consisted of a low density of attached microbial cells (10 to 10^2 per 1000 μm^2), mostly located in grain boundaries of the steel (Figure 5A,B), and consisting mainly of bacteria. Filamentous algae and diatoms were sporadically observed (mostly in spring time), with no relation to the process of ennoblement. Protozoa were rare and fungi were not observed. At the time when ennoblement of the steels (maximal mV_{sce} value) in the field (Figure 1A) was reached, the biofilms had grown dense and individual bacterial cells were no longer distinguishable (Figure 5C,D).

The conclusion is that the biofilms which grew on the stainless steel coupons ennobling in the Baltic Sea water during cold and warm seasons (Figures 4 and 5) were similar but that the ennoblement developed more slowly during the cold season.

Figure 6 shows biofilms on ennobling stainless steels exposed at 23°C to Baltic Sea water in the laboratory ecosystem. Prior to ennoblement the biofilm consisted of individual cells and small aggregates (<10 μm) sparsely distributed on coupon surfaces (Figure 6A, C and E). After the ennoblement had occurred (+300 to +430 mV_{sce}, Figure 2A) 1–10% of the steel surface had become covered by microbial aggregates (Figure 6B, D and F) similar to those observed on coupons exposed in the field (Figures 4 and 5). CTC and DAPI positive (yellow, Figure 6) areas indicate zones where sufficiently low redox potentials have developed to reduce CTC into fluorescing CTC-formazan. The figure shows that major portions of the microbial aggregate body had reduced CTC and were also stained by DAPI (DNA stain) indicating the dominance of metabolically active cells.

The CLSM images of biofilm stained with syto 16 and tetramethyl rhodamine-tagged concanavalin A (Figure 6E, F) showed microbial aggregates of a diameter up to 40 μm and thickness up to 25 μm as well as detached individual cells (Figure 6E). After 21 days one to two such aggregates were observed per 1000 μm^2 (Figure 6F). The surface layers of the microbial aggregates stained intensely in all three dimensions with the DNA stain syto-16, indicating that the aggregate was topped by densely packed bacterial cells (Figure 6F). In addition, brightly fluorescent filamentous organisms and single cells stained with cyto-16 and tetramethylrhodamine-tagged concanavalin A, were visible (Figure 6F). The filamentous organisms and small aggregates appeared positive for concanavalin A and syto 16 whereas the major aggregate was stained by syto 16 only. The compactness of the biofilm aggregates grown on ennobled stainless steel was examined using acridine orange. Figure 7, panels A–D show that acridine orange penetrated to a depth of about 10 μm into the biofilm aggregate. The interior domains of the aggregate extending 60 μm from the steel surface were dark, indicating a lack of dye permeation.

Figure 8 panels A, B and C show the microscopic structure of biofilm grown on stainless steel (UNS S31600) in N- and P-

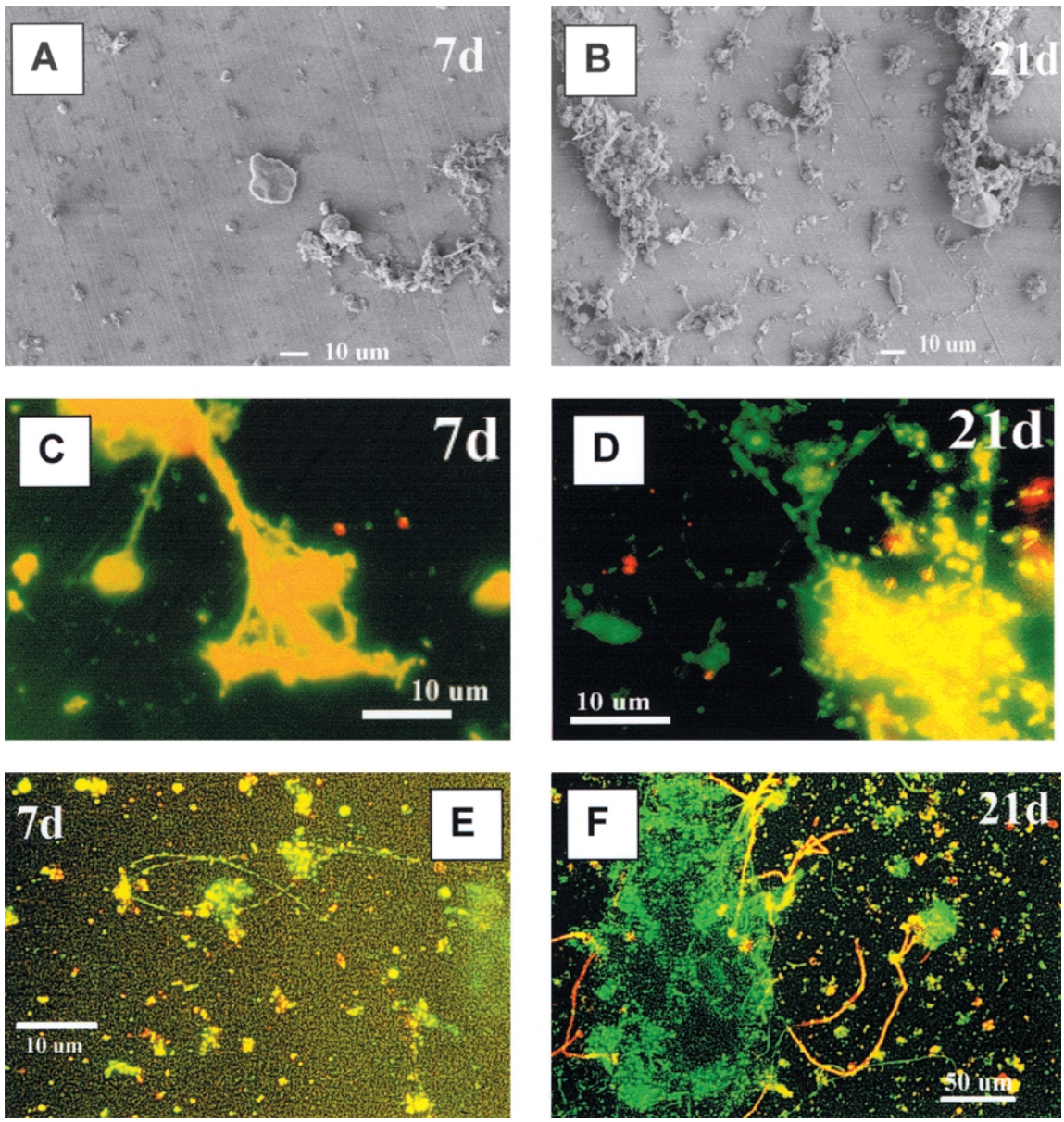

Figure 6 Microscopic structure of biofilms grown on surface of stainless steel (UNS S31600) during ennoblement in Baltic Sea water in the laboratory ecosystem at 23°C. Panels A and B show scanning electron micrographs, panels C and D epifluorescence and panels E and F CLSM images of coupons exposed as shown in Table 1, exp. L8. Open circuit potentials of stainless steel coupons increased to 335–434 mV $_{sce}$ after 10 days of exposure (Figure 2). Epifluorescence images (C, D) were taken of specimens stained with CTC and DAPI. Cells that have reached low intracellular redox potential have red fluorescence. CLSM images of biofilm stained with cyto 16 (green) and tetramethylrhodamine-labeled concanavalin A (red) are shown in E and F. Panels E and F were compiled from 35 optical sections of 1 μm each.

fertilized Baltic Sea water in the laboratory ecosystem. Figure 8 panels D and E show the structure of biofilm grown in a laboratory ecosystem in Baltic sea water accidentally enriched with N as a result of a failure in sewage treatment. The biofilms in Figure 8 differed from those shown in Figures 6 and 7 by being easily detached from the stainless steel surface. The biofilms grown in oligotrophic natural Baltic Sea water (Table 1, Figures 6 and 7) were firmly attached to the steel. Individual large (up to 1 by 5 μm, Figure 8A and B) bacterial cells were visible on the steel surface and filamentous growth was abundant compared to biofilms grown in natural Baltic Sea water (Figures 6 and 7). No clear-edged microbial aggregates were found in biofilms grown in the N-enriched Baltic Sea water (Figure 8).

Discussion

Ennoblement of stainless steel in a laboratory ecosystem

We reported earlier that ennoblement of stainless steel in Baltic Sea water was achieved in a laboratory ecosystem similarly to that achieved under field conditions [9,14]. In this paper we have

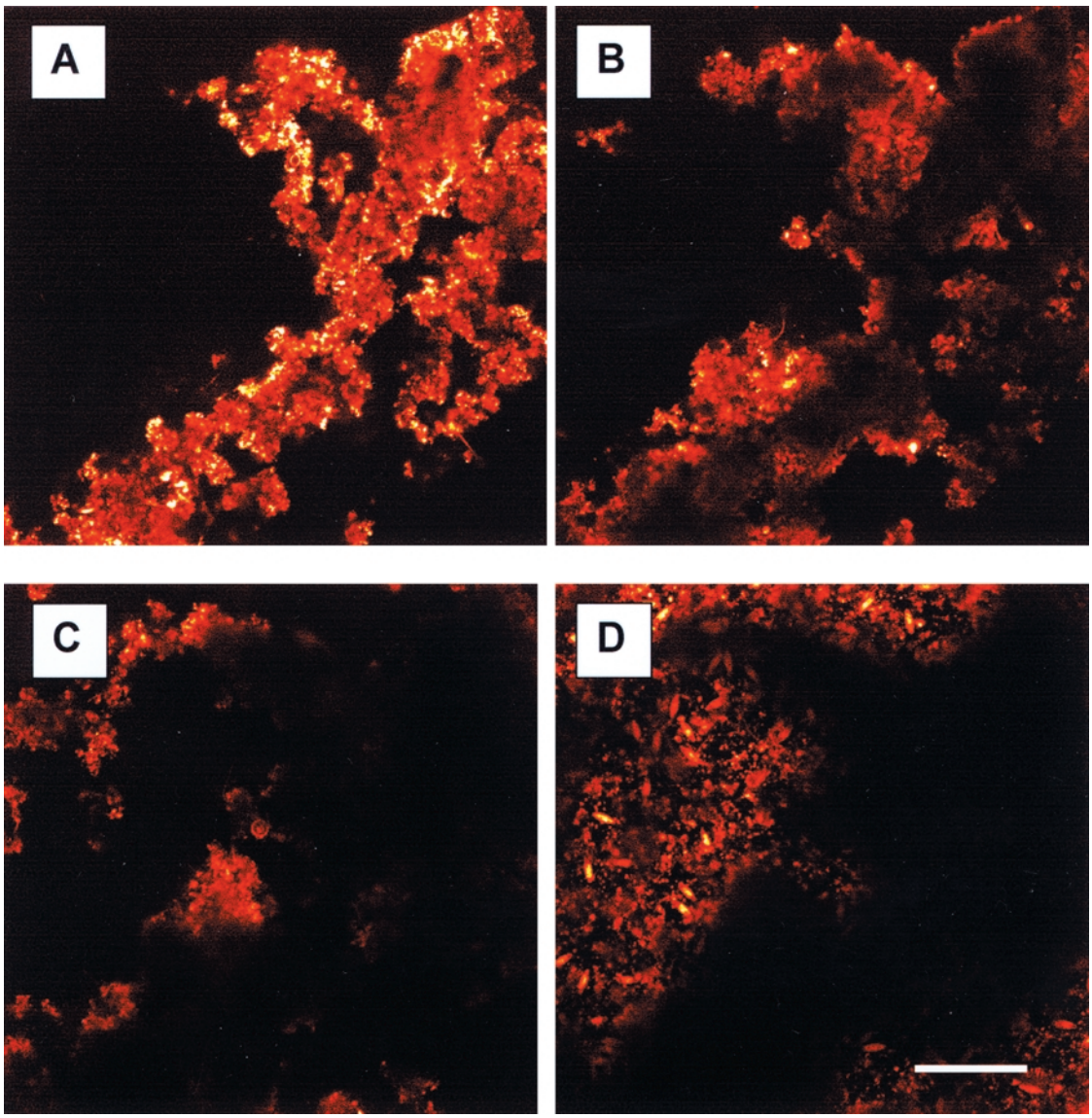

Figure 7 Microscopic structure of acridine orange-stained biofilm grown on ennobled stainless steel (UNS S31600) in Baltic Sea water in the laboratory ecosystem at 23°C. Panels A, B, C and D represent CLSM single sections, 1 μm each of the acridine orange-stained biofilm. Distance between the sections was 15 μm. Panel D shows the optical section immediately adjacent to the surface of the steel. The bar is 50 μm.

analysed the impact of environmental factors on the development of open circuit potentials of stainless steel. We found that enrichment of Baltic Sea water with nitrogen or treated municipal sewage inhibited development of ennoblement under the use of otherwise permissive conditions, such as sufficient flow rate [12,14].

The increase of the open circuit potentials of stainless steel coupons was slower at 0°C than at 23°. No low temperature threshold was found, but there was an upper limit of $\leq$32°C. For Mediterranean Sea water an upper temperature limit of 40°C has been reported for ennoblement [4,12]. Our results indicate that the biology involved with ennoblement in the Baltic Sea may operate at lower temperatures compared to that in the Mediterranean. Alabiso *et al.* [1] have shown in an experiment in the Antarctic Sea that an artificial rise of sea water temperature by 25°C over the mean local values was sufficient to strongly delay microbiological influences on the corrosion processes.

Scotto *et al.* [16], reviewed by Dexter [12], showed that steel ennoblement was lost when a respiration inhibitor, sodium azide, was added to the seawater. They also showed that no ennoblement occurred in sterile seawater. Our data indicate that ennoblement of stainless steel required not just a biofilm but biofilm with a coverage exceeding a threshold density. Our study also showed that measurement of the ATP content or the dry weight of the biofilm were not suitable tools to estimate this threshold density. It appears that ennoblement in water of low salinity (0.2–0.7%) was similar to that reported for high salinity, the Atlantic, the Mediterranean and the North Sea [6].

Our earlier observations [13,14] indicated poor permeability of ennobled biofilm aggregates to hydrophilic (concanavalin A and carboxylate-modified FluoSpheres®) and hydrophobic stains (aldehyde-sulfate FluoSpheres®) and stains having different polarity properties (acridine orange log K_{OW}1.24, ethidium

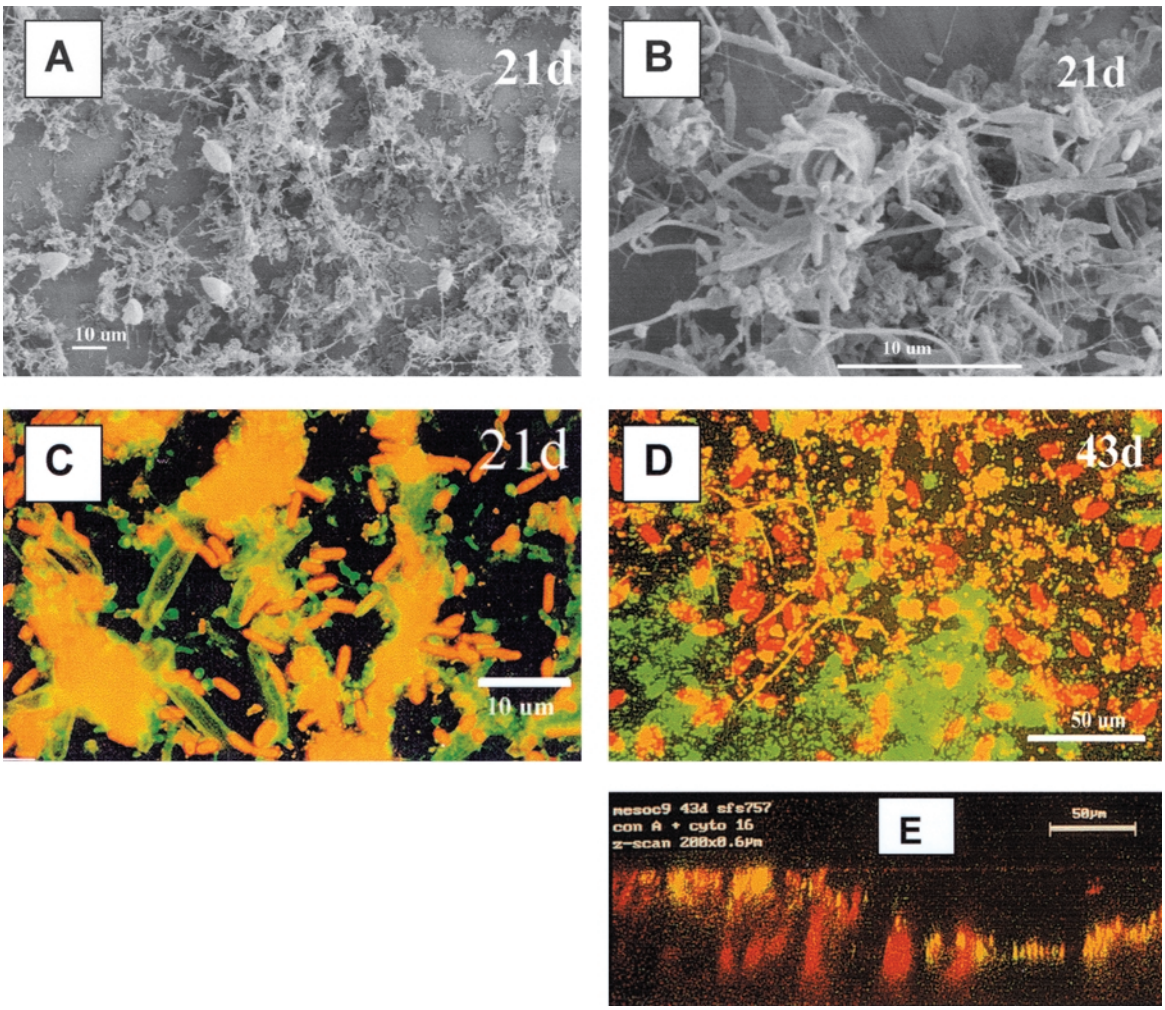

Figure 8 Microscopic morphology of biofilm grown on stainless steel (UNS S31600) immersed in fertilized (33 μg total P l^{-1} and 778 μg total N l^{-1}) Baltic Sea water in the laboratory is shown in panels A, B and C. Panels D and E show the structure of biofilm grown in the laboratory ecosystem in Baltic sea water accidentally enriched with N as a result of a failure in sewage treatment. Panels a and b are scanning electron micrographs of the fertilized 21-day-old biofilm and panel C shows an epifluorescence image of the biofilm stained with acridine orange. The CLSM image of a 43-day-old biofilm (D) is a pile-up of 85 individual *xy* scans with step of 0.6 μm stained with syto 16 and tetramethylrhodamine-labelled concanavalin A. Panel shows the vertical thickness of the biofilm (E).

bromide log $K_{OW} - 0.38$ and SYTO™ 16 log K_{OW}1.48). Our present data confirmed that the appearance of microbial aggregates with high barrier properties (against acridine orange Figure 7 and syto 16 Figure 6F) may be involved with the development of ennoblement. Costerton *et al.* [10] showed a similar barrier against oxygen.

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