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Junji AKAI[1], Katsuhiro Shimizu[1], Shogo Nakamura[2], Shoko Kuwa[3] and Ichiro Sasagawa [4]

[1] Department of Geological Science, Niigata University, Niigata 950-2181, JAPAN;
e-mail:akai@sc.niigata-u.ac.jp
[2] Department of Environmental Biology and Chemistry, Faculty of Science Toyama University, Toyama 930-8555, JAPAN
[3]The Advanced Research Center, Nippon Dental University. School of Dentistry at Niigata, Niigata 951-8580 JAPAN
[4]Department of Anatomy, Nippon Dental University. School of Dentistry at Niigata, Niigata 951-8580 JAPAN

ABSTRACT

Drifted heavy oil from the Russian tanker "Nakhodka" and related bacteria were examined by analytical electron microscope (AEM). The compositions of drifted heavy oils showed various compositions, sometimes containing metal elements etc. Transmission electron microscope (TEM) observations of bacteria growing in heavy oil-rich environments were carried out. They may be oil degradation bacteria but it is not surely confirmed because of lack of detailed microbiological examinations. There were several types of bacteria; the abundant bacteria are coccus, and bacillus and spirillum are found. At least, two types were found in ultrathin sectioned specimens: One has capsule-like structure and the other has not .

Preliminary experiments of interaction between microbes and metal elements in such heavy oil contaminated sea water environment were carried out. Heavy oil (3g), chemicals (1g) and seawater (40 ml) were put into petri dish, and after two weeks samples were observed by AEM. Bacteria living in such highly oil-contaminated and heavy metal rich environment were observed : some were containing heavy metals inside or around the bodies.

INTRODUCTION

A large amount of Heavy oil (approximately 6,240 kl of C-heavy oil) was spilled from a Russian oil tanker "Nakhodka" on 2 January 1997 in the Sea of Japan. Chemical composition of drifted heavy oil was mainly aliphatic hydrocarbons namely n-alkanes of C9-C30 n-eicosane (n-C20H42) etc. The heavy oil spill led to a serious impact to the surrounding environment. It is, in general, estimated that microbes, especially oil degradation bacteria may play an important role in such environments. In comparison to well known process of natural petroleum in reservoirs the spilled oil may also become susceptible to microbial attack. This may be aerobic or anaerobic processes. Petroleum degradation is much slower anaerobically than aerobically. Microbial attack can be used for remediation of oil. Hydrocarbon oxidation is often a strictly aerobic process but anaerobic degradation of some oil constituents are also present. Sulfate-reducing bacteria in oil-well brines are able to derive energy and/or carbon from petroleum constituents, especially methane (Panganiban et al.,1979). Kuznetsova and Gorlenko (1965) reported anaerobic attack of hydrocarbons by a strain of *Pseudomonas* in a mineral salts medium with petroleum as the only carbon source. Kvasnikov et al.(1973) obtained growth of *Clostridium* and *Bacillus polymyxa* anaerobically with n-alkanes as the source of carbon. A case of in situ microbial conversion of petroleum into bitumen has been reported from Alberta (Canada) oil sands (Rubinstein et al., 1977). However it is not always well known what fate spilled oil in the sea will have and what type of bacteria are growing. So, we examined the spilled heavy oil and also observed bacteria in the oil -rich environments. The study was carried out mainly by transmission electron microscope (TEM.). This is a basic description of the heavy oils of this type of accident.

During AEM study we found that the heavy oil is not always pure hydrocarbon but shows sometimes various compositions, such as heavy metals. This situation is also important for environmental problem. A large number of studies on interaction between bacteria and metals have been reported (e.g., Ehlrich, 1996). So, we further carried out preliminary experiments of interaction between microbes and some metals in such oil-contaminated sea water environment. Such interaction is another important aspect for understanding of natural processes and also for remediation process. Bacteria living in highly oil-contaminated environment were observed mainly under Analytical Electron

Microscope and preliminary described.

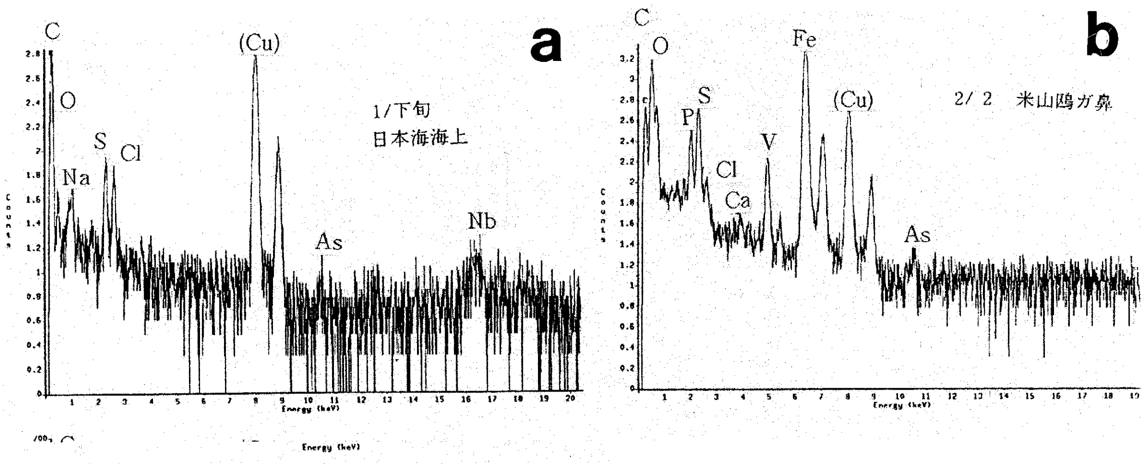
Experimental methods

We collected heavy oil samples from various sea shore (Kasashima, Yoneyama-Kamomegahana, Arahama-Sabaishi River mouth, Maki Gokahama,Oogata, Jouetsu, Ishikozawa, Niigata Kamishinmachi etc.) in Niigata Prefecture, in January and February 1997. Analysis of the oil by AEM was carried out for the part where no supporting materials are present. TEM used is JEOL 200CX, operating at 200kV. Energy dispersive X-ray spectrometer (EDS) used is Noran Inst. Voyager IV. The oil was immersed in sea water , and growing bacteria were observed by light microscope and were placed directly onto microgrid for electron microscopic observations. They were observed by TEM, 200CX with EDS Detector (Noran Instr. Voyager IV), operating at 200kV. The same specimens were further observed by the following two methods :

A) Bacteria were examined by electron microscopy, using negative staining with 1% uranyl acetate. A transmission electron microscope (JEOL 100SX) was used for observation at 80kV.

B) Seawater polluted with heavy oil was added to same quantity of Karnovsky solution (4% paraformaldehyde, 5% glutaraldehyde, 0.1M phosphate buffer:PB, pH 7.4) and fixed for two hours at room temperature. The fixed bacteria in the seawater were collected by centrifugation at 12,000rpm for 5 minutes and washed several times with 0.1M PB solution containing 0.25M glucose. Then the specimen was post-fixed with 2% osmic acid for two hours at 4 ℃ and washed with distilled water. The pellet of bacteria was suspended, embedded in 1.4% low-melt agarose and the agarose blocks was cut into small cubes. The cubes were dehydrated through an ethanol series (35%-100%), then placed in propylene oxide and embedded in ERL-Quetal 653 resin (Kushida, 1980). The resin was polymerized for a day at 60 ℃. Thin sections (70 nm) cut with a diamond knife on a Reichert Ultracut-S (Leica) microtome were placed on copper grids, stained with 3% uranyl acetate in 50% ethanol for 5 minutes, and then with lead citrate (Ultrostain2, LKB) for 10 minutes. The specimens were observed with a JEM 1200EX transmission electron microscope at 80kV.

Preliminary experiments of interaction between metal and microbe in the oil contaminated environments were carried out as follows: heavy oil(3g) with bacteria which were collected from the seashore (Kamishinmachi), various metal compounds (1g) and 40ml sea water were added into petri dish for bacteria growing experiments. The following metal compounds were used : Tested metals are Fe, Mn, Sn, Cu, Zn, Cr, Co, Cd, Sb . They are mainly chlorides form or sulfates. $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CdCl_2 \cdot 2.5H_2O$, $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $Cr(SO_4)_3 \cdot nH_2O$, $SnCl_2 \cdot 2H_2O$, $CoCl_2$. After two weeks, water samples containing bacteria were placed onto carbon coated microgrid and observed by TEM. (JEM 200CX). Operating at 200kV.



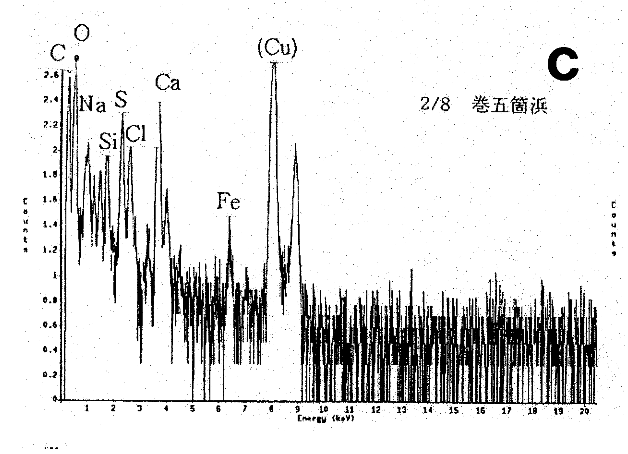


Figure 1 EDS spectra of drifted heavy oils. a) 31st January, 1997 on the Japan Sea surface ; b) 2nd Feburary, 1997 Kamomegahana,Yoneyama; c) 8th Feburary, 1977. Maki-gokahama. All vertical scales are in log scale. (Cu) indicates supporting microgrid composition.

RESULTS AND DISCUSSIONS

A AEM analyses of drifted heavy oil

Spilled oil samples were placed onto microgrids and were observed in pore parts without supporting materials of microgrid. Although they sometimes contained small solid materials, analyses were carried out on liquid parts in the TEM. Figs.1a,b

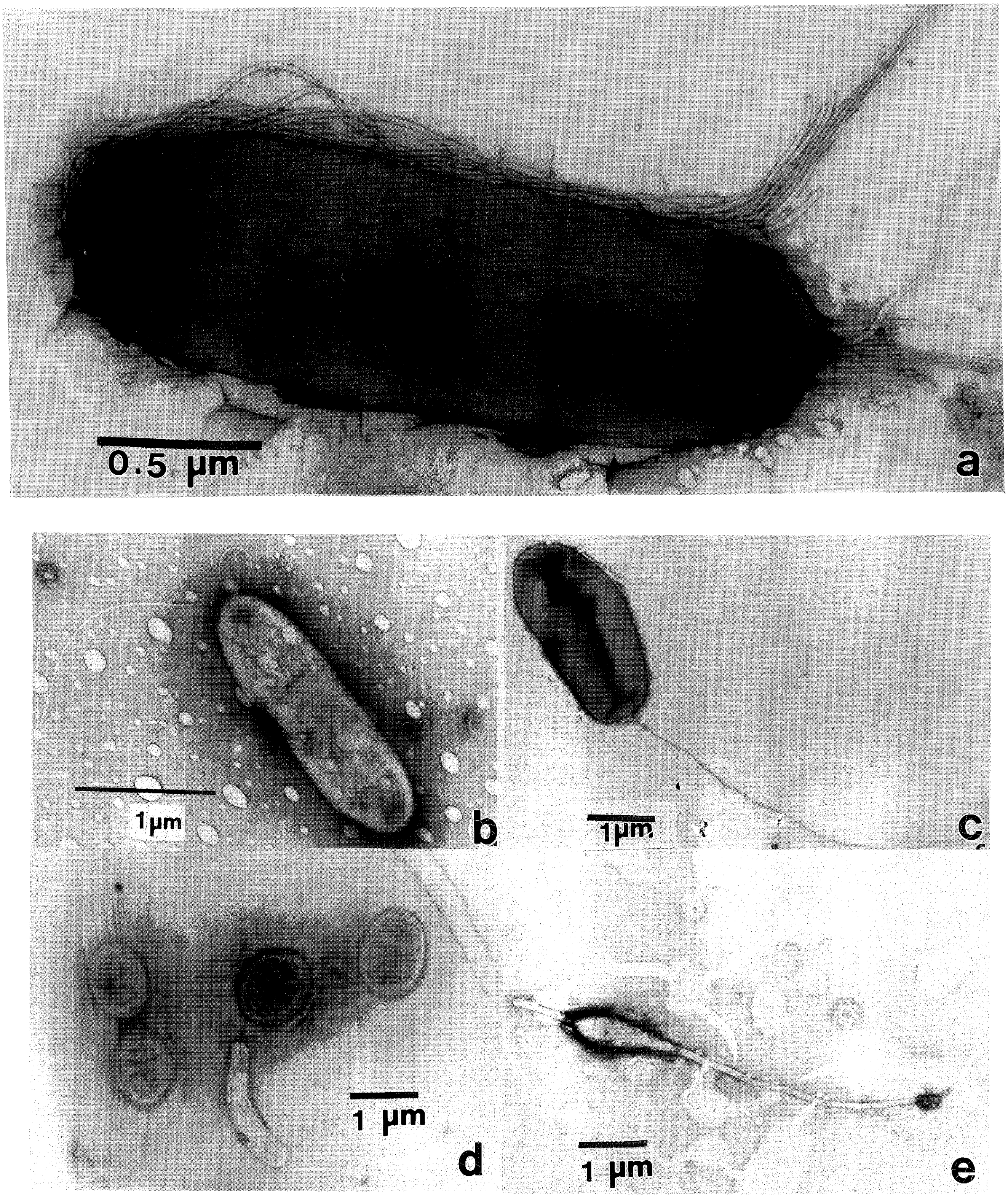


Figure 2 TEM micrographs of negative stained bacteria. a) rod shaped bacteria with flagellum; b) rod shaped bacteria with one flagellum ; c) rod shaped bacteria with a single polar flagellum ; d) coccus; e) bacterium which may be stalked one.

and c show EDS spectra indicating various compositions. Compositions of the oil is mainly carbon but sulfur etc were also sometimes found. This sulfur may be due to original heavy oil but , sometimes too much sulfur was found. In some cases other metal elements were found, such as Fe, Ca and As, etc. These results

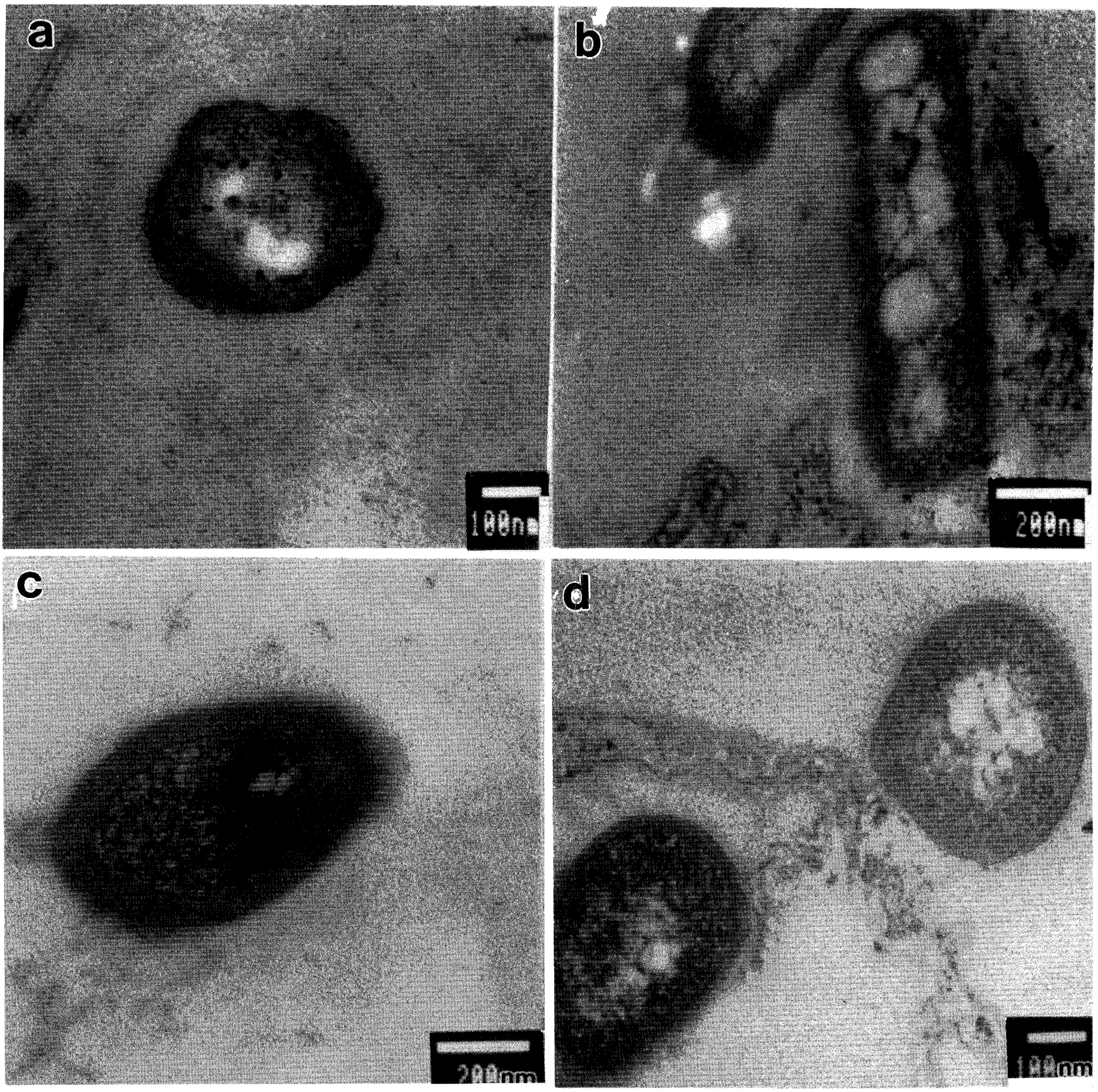


Figure 3 TEM images of ultra-thin sectioned bacteria. a) coccus; b) bacillus; c) coccus with capsule like structure; d) coccus.

indicate that heavy oil in such cases are not pure composition but contaminated with metal elements. These may be due to either some artificial sources or natural sources, such as minerals .

B TEM observations of bacteria

Spilled oil were immersed in the seawater environment. After two weeks. characteristic bacteria were living and growing in and around the spilled oil. Figs. 2 a-e show TEM images of negative stained bacteria. Fig.2a shows rod-shaped bacterium with flagella. Fig. 2b and 2c are also rod-shaped bacteria with one flagellum (a single polar flagellum). In Fig.2d bacteria are coccus. In Fig. 2e the microbe may be stalked bacterium. More information is not obtained because no detailed microbiological examinations were carried out. However, these bacteria may be degrading oil or at least coexisting with oil degradation bacteria in the same environments.

Figs. 3 a,b,c and d indicate TEM images of thin-sections of bacteria in sea water polluted with heavy oil. They show a bilayer cell membrane. Fig.3c shows bacterium with a capsule-like structure. The others show bacteria without capsule-like structure. Thus, characteristic two types of bacteria are recognized. Fig.3b shows Rod shaped bacteria.

C Interaction between microbes and metals in heavy oil contaminated sea water environment

Preliminary experiments of interaction between microbes and metal elements were carried out. After two weeks from the start of experiments , samples were obtained and examined. Figs.4 a- f show some results of the experiments. They are examples of bacteria which were collecting metal components inside or outside the bacterial cells. Fig. 4a indicates Iron (Fe) enrichment around the bacterial cell. Many bacteria coating Fe were observed in the case of Fe added conditions. Then, in the cases of Manganese (Mn) and Tin (Sn) addition, relatively many bacteria were also found. Fig.4b indicates Sn accumulations in the bacteria. In cases of addition of Copper (Cu), Zinc (Zn) and Chromium (Cr) additions, bacteria were also found. Fig. 4c shows bacteria, some of which were gathering Cupper (Cu) around the cell. Figs. 4d, 4e and 4f show the results for addition of Zn, Cr and Cadmium (Cd), respectively. However, in addition of Cd, much growing bacteria were not clearly found. These

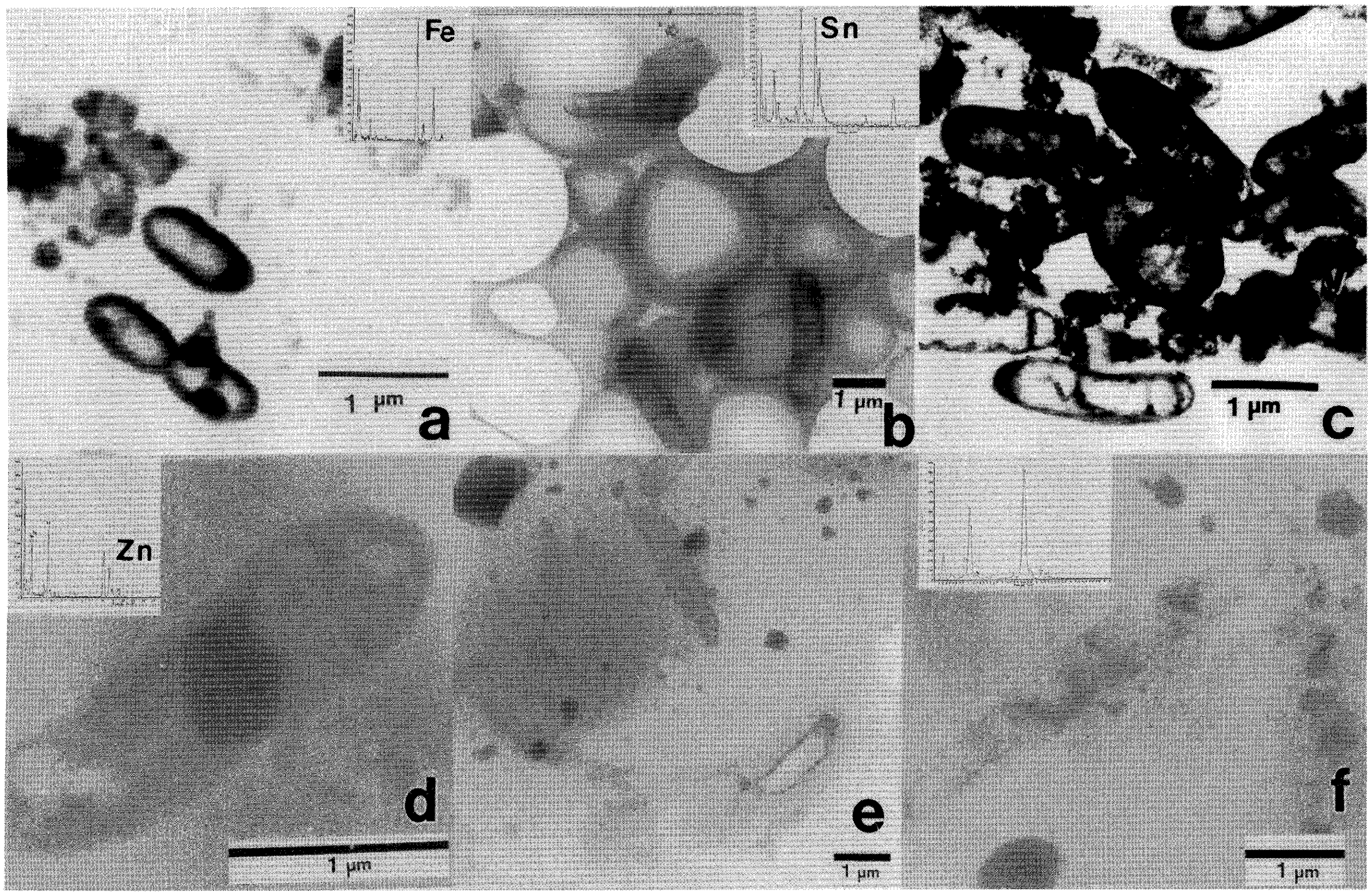


Figure 4 TEM images of interactions between microbes and various metal elements in heavy oil contaminated environments. a) Fe accumulation around the bacterial body; b) Sn ; c) Cu; d) Zn; e) Cr; f) Cd.

results may be related to metal toxicity for bacteria and to availability of metal ions for bacterial metabolism. In the latter cases, biomineralization products are sometimes formed around bacterial cells. So, the bacterial growing conditions were different depending on chemical characteristics of metal elements to bacteria. The degree of bacterial growth was largely different. It may be depending on metal's characteristics , especially their toxicity.

CONCLUSION

We described EDS analytical results of the drifted heavy oils. Sometimes, heavy oil contains various elements.

Bacteria which may be oil degradation bacteria or coexisting bacteria were observed by negative staining method and ultra-thin sectioning. Preliminary experiments of interaction between bacteria and metal elements in heavy oil contaminated sea water environments. Characteristic bacteria which are collecting heavy metals in or around the bacterial cells were found.

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