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ELECTROCHEMICAL IMPEDANCE AND DIGITAL IMAGE METHODS TO DETECT INITIAL DEPOSITION OF MICROORGANISMS

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ABSTRACT

Microorganism in industrial cooling water can cause corrosion and biofouling. Electrochemical impedance method and digital image method (DIM) were used to detect quantitatively iron bacteria and heterotrophic bacteria. Microorganism growth and metabolism can change the impedance of culture medium. The detection time of impedance variation lie in the function relationship with the logarithm of bacteria concentration. 7-million-pixel CCD sensor was used to shoot the shape of microorganism in order to count the number. Compared with most probable number (MPN) method, the detection time of electrochemical impedance is shortened. The DIM reduces the bacteria counting time and the inaccuracy associated with human count.

Keywords: biofouling, impedance, digital image processing, microorganisms

INTRODUCTION

Industrial cooling water from rivers, lakes and sea water contain lots of microorganisms which are able to grow and multiply under certain conditions when pH, water temperature and sunlight etc are suitable. One of main causes of biofouling accumulation is microorganisms' propagation which also results in accumulation of other fouling, e.g. scale deposition, particle fouling, corrosion fouling. Especially, biofouling and corrosion fouling may grow in a complementary way. Biofouling is the key cause of heat exchanger faults.

Industrial cooling water is a special and complex environment where microorganisms are suitable to grow. If microorganisms of cooling water system are excessive, sludge will deposit and plug pipe which lead to the reduction of cooling water flow and of thermal transmission efficiency. Microorganisms may cause the corrosion under fouling which result in perforation of devices in bad condition. So it is absolutely necessary to monitor biofouling in electrical power and chemical engineering factory.

Bartlett et al. designed an infra-red monitor to measure biofouling thickness accumulated on the surface of heat exchanger. Because of different thickness, infra-red absorption coefficient is different (G. Bartlett et al., 1999). The device needs to calibrate the relation of both biofouling thickness and infra-red absorption coefficient, but the calibration relation is very difficult to make for component and compactability of biofouling are not the same under different working conditions or at different time. Flemming et al. designed a FTIR flow cell based on infar-red spectrum to monitor fouling and biofouling and it can delimitate organic matter, inorganic matter, particle and biofouling. The device with the advantages of simple structure and easy control may measure quantitatively the thickness of biofouling (H. C. Flemming et al., 1998). But the disadvantage of the device is unable to realize continuous measurement in fact. Tamachkiarowa and Flemming used optical fiber sensor to monitor biofouling growth (A. Tamachkiarowa et al., 1999). When biofouling is accumulating more and more, the light intensity of reflected light will increase. At the initial stage of biofouling formation, the sensor was not sensitive enough and some factors directly affecting monitoring results such as particle diameter of microorganism, concentration and optical characteristics of the system still need studying further. Philip-Chandy and Scully et al. used an optical fiber sensor for biofilm measurement based on intensity modulation and image analysis (R. Philip-Chandy et al., 2000). The measuring results shows the sensor exhibited higher sensitive and overcame the disadvantage that is not enough sensitive at the initial stage of biofouling formation. The sensor was used on-line in site (R. Philip-Chandy et al., 1998).

We can know from the presentations above the researchers emphasized the monitoring of biofouling, but a few people research the relations of both the number of microorganisms and the accumulationg of biofouling in industrial cooling water (S.R. Yang et al., 2004). To develop quick and accurate measuring methods for microorganisms is important to study biofouling. Direct counter method is to use microscope count the microorganisms' quantity and calculate the concentration by formula. The quantity counted by direct counter method is total microorganisms. Standard of plate count (SPC) is to inoculate water samples of different dilution degrees into culture medium and place them into incubator and after a period of time, the quantity of microorganism is counted by human eyes. The lack of the method is that the incubating time of some microorganisms is too long (3-21days) and easy to miscount microorganisms. Most probable number (MPN), in essence, is like the standard of plate count. The disadvantage is also long incubating time and counting inaccuracy.

In this paper electrochemical impedance method is used to detect iron bacteria and digital image method (DIM) to quantify heterotrophic bacteria. The DIM can improve the accuracy of the SPC method, and the MPN (as an independent method) is used to confirm the results of the electrochemical impedance.

EXPERIMENTAL

Preparing of incubation media and collecting of water samples

Materials preparing incubation media selective for heterotrophic bacteria: beef extract 3.0 g, peptone10.0 g, agar 15.0 g, sodium chloride 5.0 g, distilled water 1 L. Materials preparing incubation media selective for iron bacteria: magnesium sulfate 0.5 g, ammonium sulfate 0.5 g, potassium acid sulfate 0.5 g, calcium chloride 0.2 g, sodium nitrate 0.5 g, ferric citrate 10.0 g. Water sample of 250ml was collected from Songhua River in Jilin city of China. Songhua River is a very important river in Northeast area of China and the source of industrial cooling water of lots of power plants and chemical engineering factories.

Instruments and methods

Instruments to detect heterotrophic bacteria: HR2 full automatic colony counter (Hangzhou Xunsu Ltd., China), culture dish 90 mm in diameter, biochemical incubator, scale pipette 1 mL and 5 mL, ground triangle flask 100 mL, triangle flask 500 mL, steam pressure sterilizer, electric heating drying cabinet, etc.

Instruments to detect iron bacteria: CHI660; Electrochemical operation (Shanghai Chenhua instrument Ltd. China) as shown fig. 1, platinum electrodes and the measuring structure as shown fig.2 and fig.3, scale pipette 1 mL and 5 mL, ground triangle flask 100 mL, ground reagent bottle 1000 mL, volumetric flask 1000 mL, test-tube 150 mm × 15 mm, test-tube stand, biochemical incubator, steam pressure sterilizer, electric heating drying cabinet, etc.

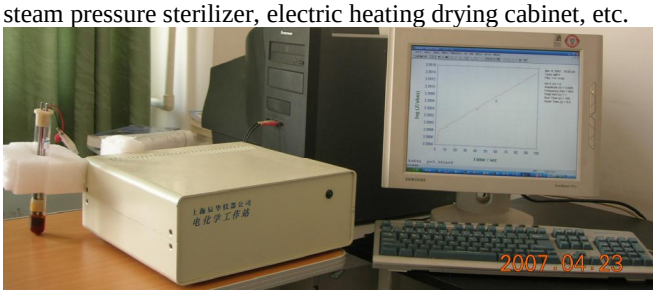


Fig.1 Experimental impedance setup

Fig. 2 Platinum electrodes

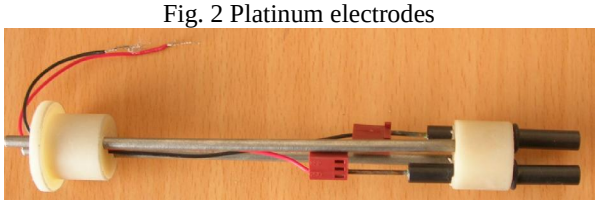


Fig. 3 Measuring structure of platinum electrodes

During the process of growth and metabolism of microorganisms , chemical constitution in culture medium may generate change, which can be measure by measuring impedance, because both nutrient substances are been depleting and outgrowth of metabolism are accumulating more and more. Two platinum electrodes were inserted into culture fluid and sine wave electronic signal was put in the platinum electrodes, the change of impedance signal was recorded with the time change. So microorganisms' growth information was gained by the impedance signal. z_0 is the conductivity of the culture medium at the beginning of test and z is the conductivity of the culture medium at the testing process. If the relative magnitude of the conductivity's change reaches 4.0 %, there is function relation between the reaching time t_d, i.e. the detecting time, and the microorganism concentration at the initiative time. The lower the initiative microorganism concentration of water sample is, the longer the t_d is.

We use Impedance – Time (IMPT) method to measure impedance. The base potential is held constant at Init E. A sine waveform is superimposed to the base potential. The current and the potential are sampled and analyzed to obtain the real and imaginary impedance. The impedance is recorded as the function of time. Fig.4 shows the potential waveform applied as the function of time. Quiet time is 0.5s. Run time is 100s. Initial potential is 5V.Amplitude of AC voltage is 5 mV. Frequency is 1 kHz. Sample interval is 1s. These parameters are able to be set in the software of the electrochemical operation, CHI Version 6.25. The measurement results of impedance can be recorded real time and the curves of results were shown on the screen. Scheme of experimental setup is shown in fig.5. Two platinum electrodes in culture media was connected with electrochemical operation, connected with PC computer.

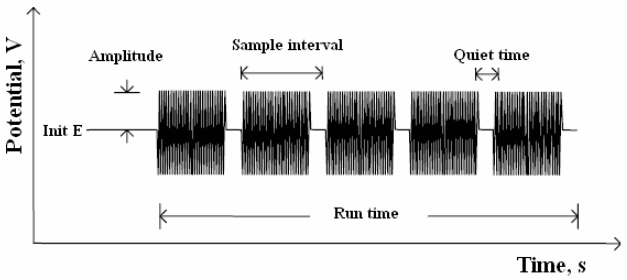


Fig. 4 Principle diagram of impedance method

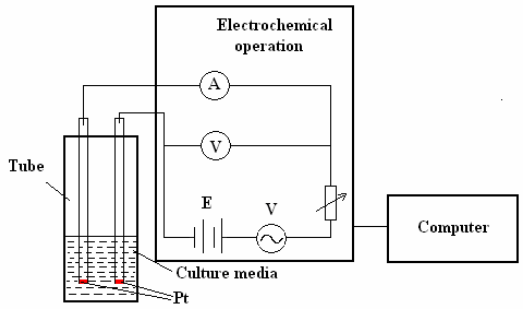


Fig.5 Scheme of experimental setup

Iron bacteria of the water sample in Songhua River was selected as the detecting object, liquidness beef extract peptone being culture medium, under the test condition of at 29±1 ℃, t_d of water sample with different microorganism concentration was gained. At the same time the control test was made by MPN method which the results of microorganism concentration were used to calibrate the t_d. In fact, td and initiative concentration of microorganism exit quantitative relation, which is the basis of microorganism detection by electrochemical impedance method.

SPC is the standard method to detect heterotrophic bacteria, but the disadvantage of the method is its inaccuracy. DIM is used to count heterotrophic bacteria in the experiment; the advantage is its counting accuracy and quickness than human eye count. A CCD sensor with 7-million-pixel was used to shoot the growth of heterotrophic bacteria. Many parameters may be set in the image processing software like threshold value of magnitude of heterotrophic bacteria, shade of gray, 0.1 mm in diametric and 0.2 in circular degree coefficient. Water samples with different dilution degrees were cultured. After 72 hours all culture dishes were shot to be counted according to the setting parameters. There are three culture dishes in every dilution degree. We used the same 3 plates to perform the SPC and DIM measurement.

Experimental process

Water samples were diluted to different multiples and definite volumes were taken out to mix with incubation media in test-tubes. Every dilution degree had four parallel culture tubes, three of which are used as MPN method and the other one is used as impedance method. At 29℃, mixtures were cultured 14days. At last, if there was black or brow deposition yielded and brow culture media vanished in the tubes, it shows there is iron bacteria, marked by positive signal "+", or else "-". According to the positive signal association mark, MPN of iron bacteria is achieved by look-up table. The result that the MPN divided the dilution degree of first number of the positive signal association mark is the number of iron bacteria per milliliter.

The process of impedance method is to take out several milliliter water samples to culture medium and to place them into incubator to measure the impedance change with the increase of time. Measurement order is from low (10^{-5}) to high dilution degree (10^{-1}). After the impedance of culture media in every tube was measured, the platinum electrodes were washed using ethanol and distilled water and dried by high temperature sterilizing oven. One measuring time for impedance is 100 s, every tube is measured 4 times and the impedance average of 4 times measured results was as the record impedance value of this dilution degree tube of this day.

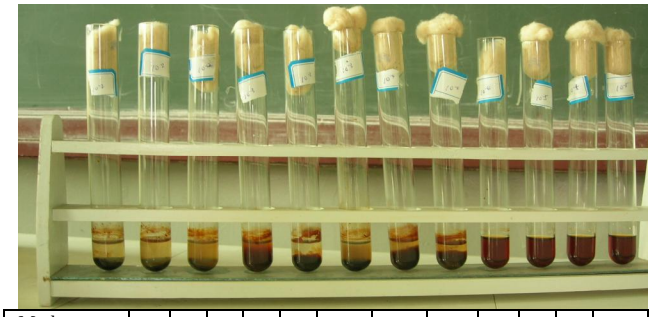
The chief procedure of standard of plate count and digital image counting method is to dilute water sample to different multiples, to take out definite volume water to mix uniformly with incubation media and to paint on culture dishes. In the incubator at 29℃ mixing water samples were cultured for 72 hours. At last, the colony numbers were counted. The mean value of three parallel culture dishes was regarded as the results of standard of plate. At the same time all culture dishes were shot to count and the mean value of three parallel culture dishes was as the results of digital image counting method.

RESULTS AND DISCUSSION

Iron bacteria

The culture results for iron bacteria are shown as fig. 6 which indicates the positive signal association mark is 320. By look-up table and divide-dilution ratio, the number of iron bacteria in raw water sample is 9500 cfu/mL. The water sample was collected on March 24th, 2007 and the temperature of river water and outside was respectively 7℃ and 2℃ above zero. We once collected another water sample at the same site of Songhua River on January 13th, 2007 and the measurement result of iron bacteria was 950 cfu/mL. In January it was winter, the temperature of river water was 4℃ above freezing and the temperature outside was 20℃ subzero (PS: the areas of Songhua River through Jilin city never freezes). With the increment of temperature, the numbers of iron bacteria in Songhua River also goes up. Cooling water from Songhua River includes many iron bacteria, which is unavoidably to bring biofouling in pipes. But the disadvantage of MPN method is the detection time

is too long, thus it's inconvenient to monitor the changes of iron bacteria.



Mark	+	+	+	+	+	+	+	+	-	-	-	-
Dilution degree	10^{-2}			10^{-3}			10^{-4}			10^{-5}		
Association mark				3			2			0		

Fig.6 The culture results of iron bacteria after 14 days

Electrochemical impedance method

In order to short the detection time we used the electrochemical impedance method to measure the iron bacteria. Microorganism growth has definite rule, which can be describe by growth curve as shown in fig. 7 (L. Cen et al., 2004). The stages in turn are lag phase, exponential growth phase, stationary phase and decline phase. In the lag phase, microorganism hardly grow, the reason is microorganism need a period time to adapt the culture media environment. After a period time, microorganisms began to split, reproduce and grow with geometric progression; this period is called exponential growth phase in which the grow speed is maximal. When nutrient substance were almost consumed to nothing, the grow speed gradually decrease, and the splitting speed of microorganisms also decrease while the dead rate is gradually rising, at last the dead rate and reduction rate is almost equal. Live microorganisms keep stable in this period which is called stationary phase. When the dead rate is higher than reduction rate, live microorganisms are gradually reducing and it arrives the decline phase in which live microorganisms decline with geometric progression.

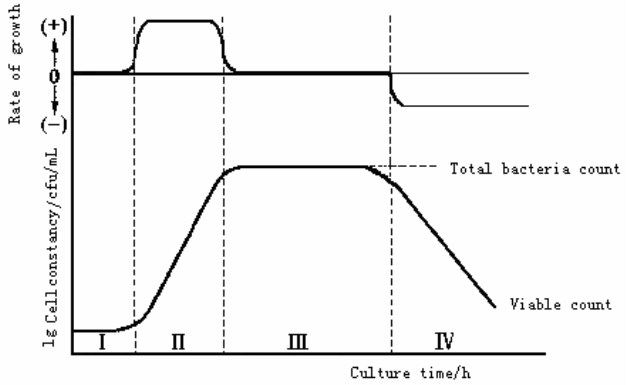


Fig.7 Microorganism growth curve log graph

In fact, impedance curve of iron bacteria, as shown in fig.8, is reverse with the growth curve of it. At the beginning stage, iron bacteria propagate quickly, metabolism of iron bacteria also improve and the impedance decreases accordingly; after 5 to 6 days the impedance began to increase which shows iron bacteria have not already grown and in the tubes brown deposition began to appear. After about 10 days in the bottom of the tubes brown deposition emerged thoroughly and the upper side of the tubes the liquid change to transparent. The platinum electrodes were inserted into the transparent liquid of the tubes to measure impedance which have hardly changed. We measured two dilution degrees of 10^0 and 10^{-1} samples. Now we had only tested a few water samples, so we can not obtain the fitting curve of impedance vs detection time. We hope to test water samples of Songhua River for one year, which is able to know the whole arrange of iron bacteria number and obtain the relations between impedance and detect time. Many researchers had acquired the linearity equation about other bacteria (Press et al., 1994; Pugh et al., 1998; Fomter, 1996).

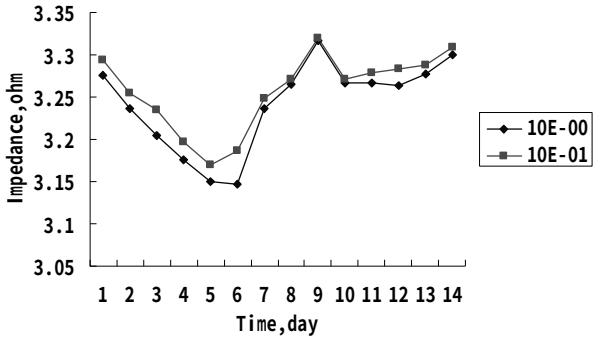


Fig. 8 Impedance curve of two dilution degree of 10^0 and 10^{-1} for iron bacteria

Heterotrophic bacteria

After Water samples were collected, different dilution degree were selected which must guarantee the colony counting was lower than 300 when the last dilution degree was inoculated. At every dilution degree three plates were used simultaneously to culture. At last, mean values were calculated. We counted the colonies with dilution degree of mean value between 30 and 300 and according to the National Bureau of Standard of China GB/T 14643.1-93 the counting results, as shown table 1, were obtained.

Table 1 Measurement results of standard of plate count and digital image method

method	dilution degree and colony count (mean valve of 3 plates)			Report results (cfu/mL)
	10^{-1}	10^{-2}	10^{-3}	
standard of plate count	-	289	35	3.2×10^{4}
digital image method	1441	362	154	3.6×10^{4}

Digital image method

The deficiency of the SPC is that counting result is probably lower than the number of normal exiting living cells, because microorganisms can exit in the form of single, coupled, chain, cluster or agglomeration and no any culture media can meet the physiological need of all bacteria in one water sample. In addition, the counting time is rather long, and it needs to be counted manually, which easily results in miscounting. So we proposed to use digital image method to count heterotrophic bacteria. A suitable threshold must be selected, in the experiment we set 0.2 mm as the resolution of count. From Table 1 we can find there is a big difference on the results for colony count on the 10^{-3} dilution degree using DIM and SPC method, the reason is that in the dilution degree solidified culture medium produced non-uniform and formed many big and sub-transparent blocks which lead to miscounting. But these blocks are able to be picked out by human eyes, and the results (154) measured by DIM are higher about one magnitude order than by SPC. At other dilution degrees, culture mediums of plates don't form non-uniform and many blocks, so the results are creditable. The DIM method need to use more and appropriate algorithms to filter image noise and improve the accurateness of classifying rules. The measurement instrument and software interface are shown as in fig. 9.

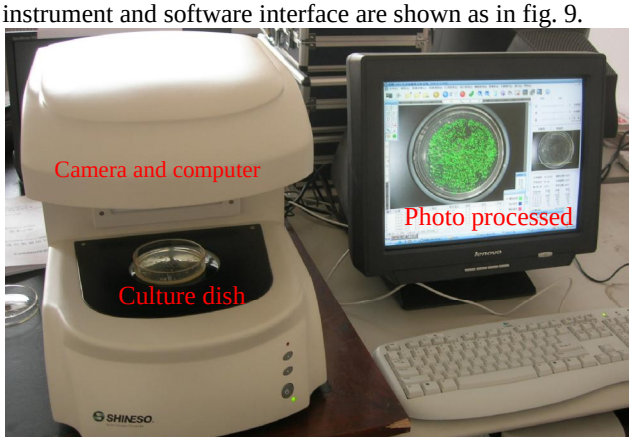


Fig. 9 Measurement instrument based on digital image method

CONCLUSIONS

Electrochemical impedance method is used to detection iron bacteria, which shorts the detect time. Digital image method is used to count heterotrophic bacteria, which improves the accuracy of counting.

Many factors such as microorganisms' concentration, temperature, pressure, velocity of flow, organic matter, pH and some inorganic matter affect the formation of biofouling, but the initial concentration of microorganism in cooling water and the growth speed of microorganism are the most important factors for biofouling formation. If the growth speed of heterotrophic bacteria is able to be measured by digital image method and the growth speed of iron bacteria by impedance method every day, and if the running conditions of cooling water are known, then it is possible that the biofouling formation in the environmental conditions is able to be predicted by the equation of initial biofouling formation proposed by W. G. Characklis (W. G. Characklis, 1981), which is significant to take some preventive measures.

NOMENCLATURE

t_d detect time, h or s
z_0 conductivity of culture medium, s
z conductivity in test process, s

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