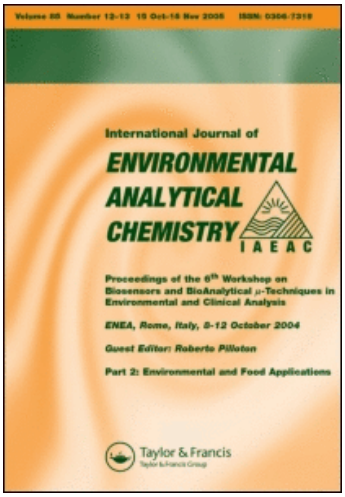

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Quartz crystal assay for rapid determination of nitrifying bacterial cells

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A quartz crystal analysis (QCA) system was developed for the rapid and simple detection of nitrifying bacteria. This method is based on the fact that nitrifying bacterial cells that reacted with anti-nitrifying bacteria antibodies were able to be analysed by QCA. Latex beads immobilized with the antibody were used for the selective detection of the nitrifying bacterial cells. A suspension of the latex beads was added to the tube chamber of the quartz crystal and incubated for 5 min at 37°C. When the suspension of nitrifying bacterial cells was injected in the chamber, the resonant frequency decreased with increasing cell concentration within 1 min. For the reference bacterial cells, the resonant frequency did not change. A good correlation was observed between the change in frequency ($-\Delta F$) and the bacterial concentration. One measurement could be completed in 10 min.

Keywords: Environment; Bacterial detection; QCA; Immunoassay; Water

1. Introduction

Nitrifying bacterial cells such as *Nitrosomonas* sp. and *Nitrobacter* sp. play an important role in many environments including soils, sewage treatment plants, fish tanks equipped with biological filtration, etc. [1]. The nitrogen cycle includes the decomposition of nitrogenous organic residues (fish wastes, uneaten foods, and dead plant materials) into the ammonium form. Ammonia is converted into nitrite and then into nitrate by the nitrifying bacterial cells. The susceptibility of fish to high concentrations of nitrogenous compounds such as ammonia and nitrite is extremely high. In general, when the number of nitrifying bacterial cells is above 10^7–10^8 cells mL^{-1} determined by the microtiter technique, the desired decomposing effect was obtained [1]. It is therefore necessary to understand the presence of a sufficient mass

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for maintaining the water quality. A colony counting method has been used for the detection of bacterial cells [2]. However, this method is time consuming and some nitrifying bacteria species cannot develop into colonies on agar medium. Therefore, the establishment of a more rapid procedure for the detection of nitrifying bacterial cells has long been required for water monitoring.

In a previous study, we developed a new system for the rapid detection of nitrifying bacteria using the photon correlation spectroscope (PCS) technique [3]. The method was based on the reaction between nitrifying bacterial cells with latex beads covered with anti-nitrifying bacterial antibodies. When nitrifying bacterial cells were reacted with the antibody beads, the size distribution was shifted to the higher range. In contrast, in the reference bacterial cells the size distribution showed little change. The phenomenon was assumed since the antibody selectively reacted with nitrifying bacteria, became aggregated with the bacterial cells, the distribution size increased. Thus, it is possible to discriminate between the nitrifying bacteria and other species of bacterial cells using the PCS technique. One assay could be completed within 60 min. However, this method required a complicated procedure and equipment.

In recent years, the use of the quartz crystal analysis (QCA) technique has remarkably progressed. A quartz crystal has been used as a mass detector and has been applied to determine gases, ions and immuno-compounds [4–8]. These studies are based on the fact that the resonant frequency change of the quartz crystal corresponds to mass change on the crystal surface [9]. In addition, we reported that the resonant resistance of the quartz crystal shows the energy loss of the vibrating quartz crystal, therefore the resonant resistance reflects the viscosity on the quartz crystal surface [10]. We have applied the resonant resistance measurement for monitoring fermentation processes, the coagulation assay of endotoxin, etc. [11, 12]. For the fibrinogen coagulation assay, the sedimentation of polystyrene beads was used for amplification of the changes in the resonant frequency during the coagulation [13]. Furthermore, the resonant resistance was used for characterizing thin films on the quartz crystal surface by diagramming the resonant frequency and resonant resistance [14]. By this method, the mass change and viscoelastic change on the quartz crystal can be analysed. The phase transition of the Langmuir–Blodgett films and polymerization process of epoxy resin have been studied using this method [15, 16]. The diagramming method of the resonant frequency and resonant resistance is an effective method to characterize thin films or deposition layers on the quartz crystal. Since the QCA possesses advantages such as simplicity and portability, its use for the detection of the nitrifying bacterial cell in aquatic environments was investigated. In this study, we developed a new system for the rapid and simple detection of nitrifying bacteria using the quartz crystal and an immuno-reaction.

2. Experimental

2.1 *Reagents*

The bonito extract was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Peptone was purchased from Difco Laboratories (Michigan, USA). The anti-*N. europaea* rabbit antibody was obtained from Yakuluto Honsha Co. Ltd

(Tokyo, Japan). All other reagents used for the experiments were commercial and laboratory grade.

2.2 *Microorganisms and cultivation*

Nitrosomonas europaea NBRC 14298 was obtained from National Institute of Technology and Evaluation (Chiba, Japan) as a model of nitrifying bacteria. This strain was cultured in a medium consisting of ($g\,L^{-1}$): $(NH_4)_2SO_4$ (0.5), NaCl (0.3), K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.3), $FeSO_4 \cdot 7H_2O$ (0.03) and $CaCO_3$ (7.5). *Paracoccus dinitrificans* IAM 12479 was chosen as the reference microorganism, and obtained from the culture collection at the Institute of Molecular and Cellular Biosciences, University of Tokyo (Tokyo, Japan). This microorganism was cultivated in EBP agar which contained ($g\,L^{-1}$) the bonito extract (3.0), peptone (5.0), NaCl (3.0) and agar (20.0). Each strain was incubated at 25°C in a shaker, indicating cell numbers of 10^5–10^7 cells mL^{-1}.

2.3 *Preparation of bacterial sample*

Ten millilitres of each cell culture were transferred to a test tube, precipitated by centrifugation (1,500 *g*, 30 min), and re-suspended in 10 mL of 0.3% formaldehyde at 5°C for 12 h. Following incubation, the cells were washed (8,500 *g*, 2 min), pelleted, and resuspended with 0.1 M phosphate buffer solution (PBS) (pH 7.8). The concentration of each cell species was adjusted to 10^4–10^6 cells mL^{-1} using the PBS.

2.4 *Apparatus and assay procedure*

The QCA system was composed of the quartz crystal fixed in a tube chamber, an oscillating circuit, an analyser (QCA917 Seiko EG&G, Japan) and a personal computer. A schematic diagram of the system is shown in figure 1. An AT-cut quartz crystal

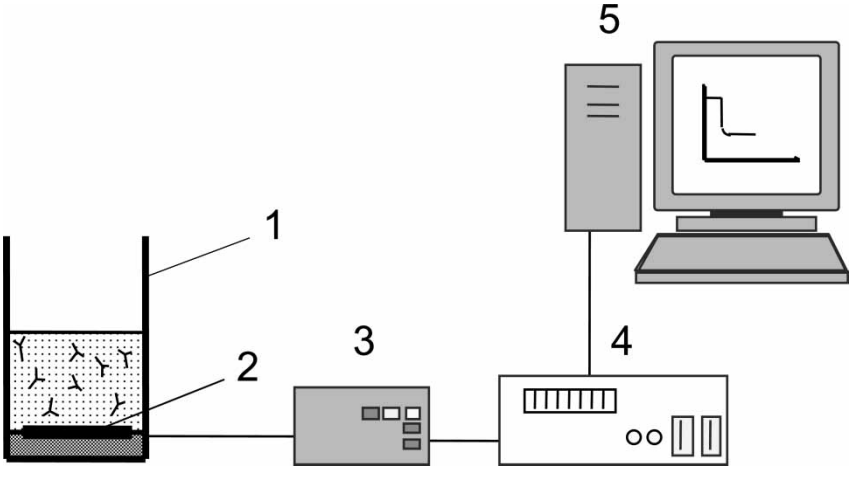

Figure 1. Schematic diagram of the QCA system for the detection of nitrifying bacterial cells. (1) tube chamber, (2) quartz crystal, (3) oscillating circuit, (4) QCA analyser (QCA917), (5) personal computer.

possessing a basic resonant frequency of 9 MHz was used. The quartz crystal was fixed to the chamber with silicon sealant in which only one side of the quartz crystal electrode (platinum) was allowed to contact the liquid. The quartz crystal is located at the bottom of the chamber so that the sedimentation can be monitored as sediment on the quartz crystal surface. The resonant frequency and resonant resistance of the quartz crystal were continuously monitored on the computer display and saved to the hard disk for later analysis. For the selective detection of the nitrifying bacterial cells, latex beads (size: ca. 1 μm) immobilized with anti-*N. europaea* rabbit antibody (titer: 4×10^4 cells mL^{-1}) were used. They were specially prepared by Yakuluto Honsha Co. Ltd (Tokyo, Japan). A 100 μL suspension of the latex beads (10^{10} pieces mL^{-1}) was added to the tube chamber of the QCA, and then the resonant frequency and resonant resistance were monitored at 37°C. When the resonant frequency and resonant resistance changes were constant, 50 μL of the bacterial sample was continuously added to the tube chamber of the QCA. The presence of bacterial cells was detected as variations in the resonant frequency and resonant resistance.

3. Results and discussion

3.1 *Typical response of the QCA system*

Figure 2 shows a typical response of the QCA system for nitrifying bacterial cells (*N. europaea*). In figure 2(1), the horizontal axis represents the measured time of the QCA system and the vertical axis represents the resonant frequency of the

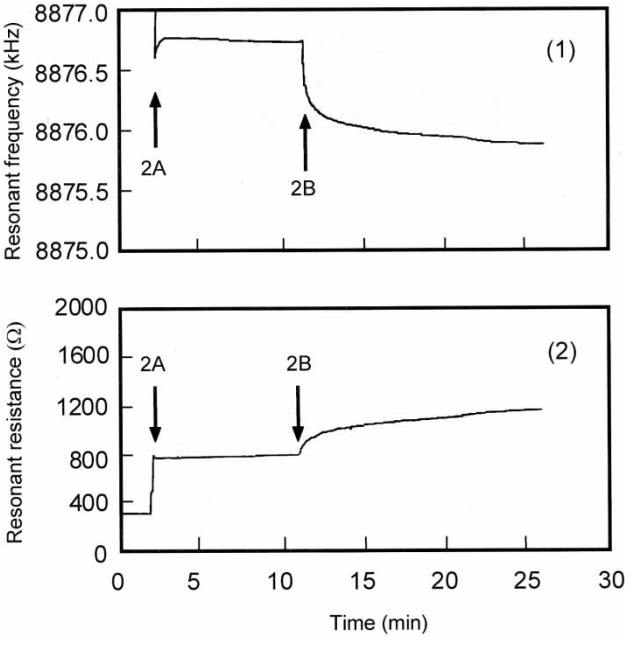

Figure 2. Response curve of the QCA system for nitrifying bacterial cells (*N. europaea*). (1) time course of resonant frequency, (2) time course of resonant resistance, (2A) injection of the latex beads, (2B) injection of the cell suspension.

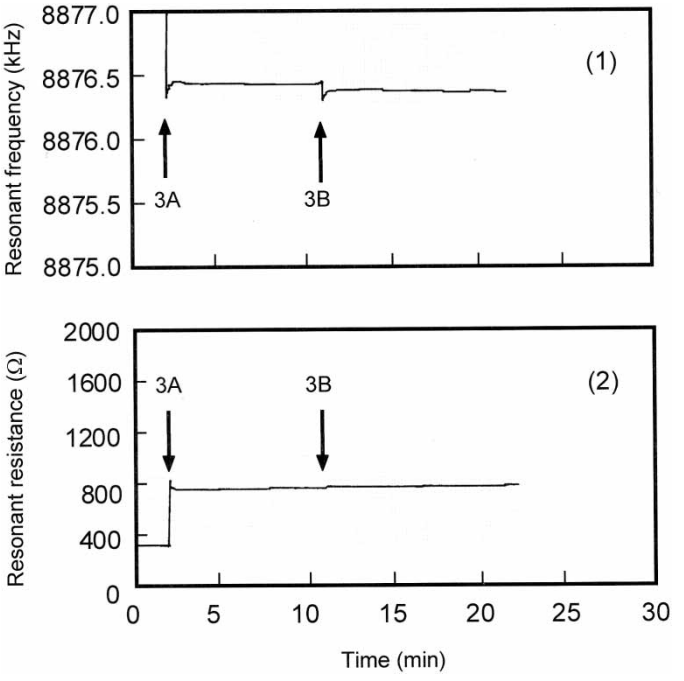

Figure 3. Response curve of the QCA system for reference bacterial cells (*P. dinitrificans*). (1) time course of resonant frequency, (2) time course of resonant resistance, (3A) injection of the latex beads, (3B) injection of the cell suspension.

quartz crystal. The suspension of latex beads (100 μL) was injected into the tube chamber of the quartz crystal (arrow 2A) and incubated for 5–7 min at 37°C. The resonant frequency became constant within 1 min and subsequently decreased. After the incubation, 50 μL of the cell suspension (*N. europaea*, 1.6×10^6 cells mL^{-1}) was injected into the tube chamber (arrow 2B). The resonant frequency significantly decreased for a few tens of seconds and then, gradually decreased. In figure 2(2), the resonant resistance of the quartz crystal is plotted along the vertical axis on the same bacterial sample measurement. The resonant resistance gradually increased after injecting the latex beads (arrow 2A). When the suspension of *N. europaea* cells (50 μL) was injected, the resonant resistance significantly increased for a few tens of seconds and then gradually increased.

The responses of the QCA system for the reference bacterial cells (*P. dinitrificans*) are shown in figure 3. The horizontal and vertical axes represent the same parameters as in figure 2. Both the resonant frequency and resonant resistance show little change when the cell suspension of *P. dinitrificans* was injected into the tube chamber (arrow 3B).

3.2 *Mechanism of sedimentation based on the diagram of the resonant frequency and resonant resistance*

As we have previously reported, films and sediments on the quartz crystal can be characterized by diagramming the resonant frequency and resonant resistance for

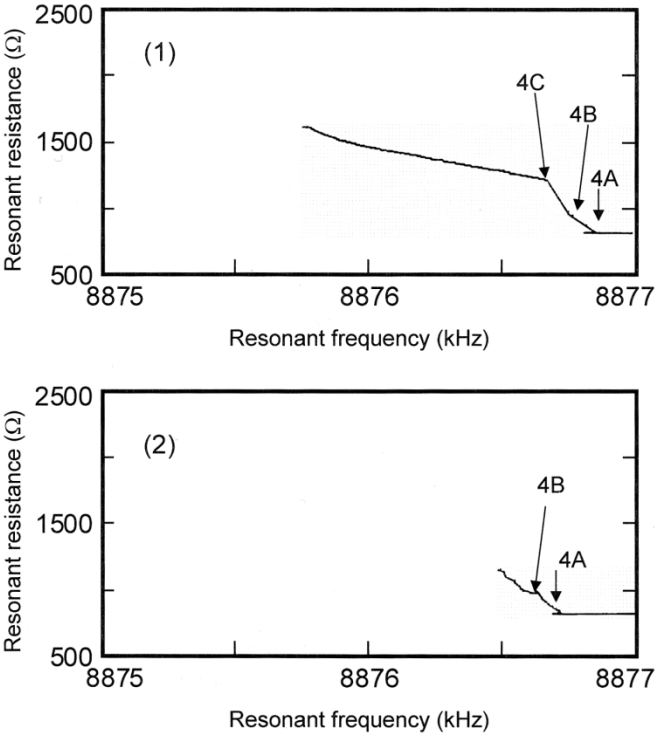

Figure 4. Diagram of the resonant frequency and resonant resistance for bacterial cells. (1) nitrifying bacterial cells (*N. europaea*), (2) reference bacterial cells (*P. dinitrificans*), (4A) injection of the latex beads, (4B) injection of the bacterial cells, (4C) inflection point.

their viscoelastic properties [15, 16]. When an elastic film is loaded on the quartz crystal, only the resonant frequency decreased while the resonant resistance does not change. When the viscoelastic film is loaded, the resonant frequency decreases and the resonant resistance increase with its viscous property. Based on the slope, the viscous property can be monitored and differences in the films on the quartz crystal can be distinguished.

Figure 4(1) shows the diagram of the resonant frequency and resonant resistance for figure 2. A proposed illustration of the sedimentation layers in the tube chamber of the quartz crystal is also indicated in figure 5. In figure 4(1), there are three regions showing different slopes after injection of the latex beads (arrow 4A). The first region is just after injection of the latex beads (arrow 4A) up to the injection of the nitrifying bacterial cells (arrow 4B). This region shows sedimentation of the latex beads because only the latex beads are present in the chamber (figure 5(5a–5c)). After the injection of the nitrifying bacterial cells (arrow 4B), there are two slopes divided by the inflection point (arrow 4C). This shows that there are two different sedimentation processes existing after the injection of the nitrifying bacterial cells (figure 5(5d–5e)).

The slope between the point 4B and 4C appeared within 20 s. This is a very rapid response. The slope of resonant resistance *versus* resonant frequency for region 4B–4C is greater than that in region 4A–4B. This indicates that the sedimentation layer for the range 4B–4C is more viscous than the layer made by the latex beads

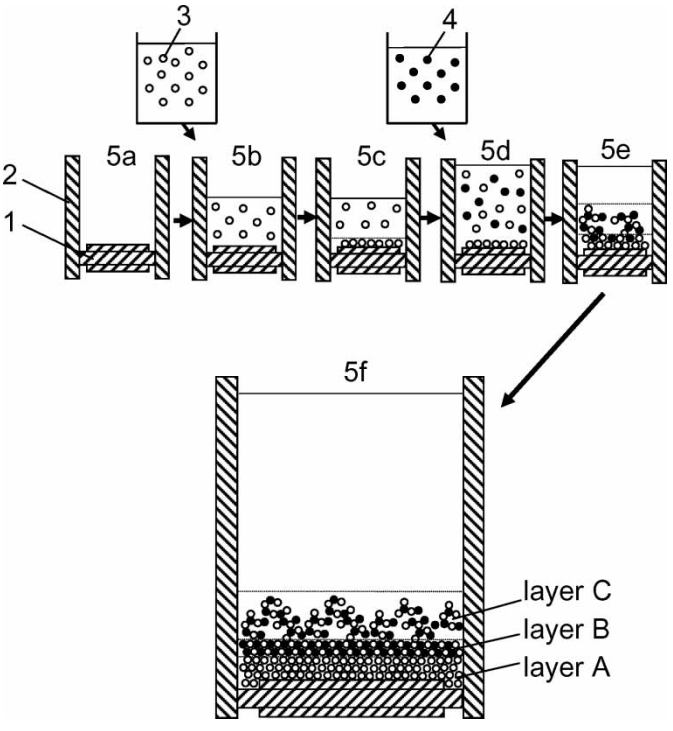

Figure 5. Illustration of sedimentation layers in the tube chamber of the quartz crystal. (1) Quartz crystal, (2) tube chamber, (3) latex bead, (4) bacterial cells.

only (region 4A–4B). In addition, as shown in figure 3, only little frequency change was observed when the reference bacteria cells were injected into the chamber. Therefore, the layer for 4B–4C should be a densely mixed layer of latex beads and nitrifying bacterial cells.

After the inflection point 4C, the slope of the resonant resistance *versus* resonant frequency is gradual and the time for this region was 15 min. This indicates that the immuno-complex of latex beads and nitrifying bacterial cells slowly sedimented. Thus, the sediment layer is not dense, but the binding of the latex beads and nitrifying bacterial cells is tight because the gradual slope means a higher elastic property compared with the other sedimentation regions in figure 4(1). Therefore, the sedimentation layers can be illustrated as in figure 5(5f) where layer A is the latex beads layer, layer B is the densely immune-complex layer and layer C is the porous but tight immuno-complex layer.

Figure 4(2) is a diagram of the resonant frequency and resonant resistance for the reference bacterial cells. Point 4A and 4B are the injection points of the latex beads and reference bacterial cells, respectively. In this figure, the slopes of region A–B and region after B are very similar. Therefore, the sedimentation layers for both range 4A–4B and after point 4B were concluded to be made by the latex beads, and no immuno-complex was deposited on the quartz crystal surface. It can be concluded that the resonant frequency change for the region where the slope in the diagram of the resonant frequency and resonant resistance is different from that of the latex beads corresponds to the immuno-complex amount.

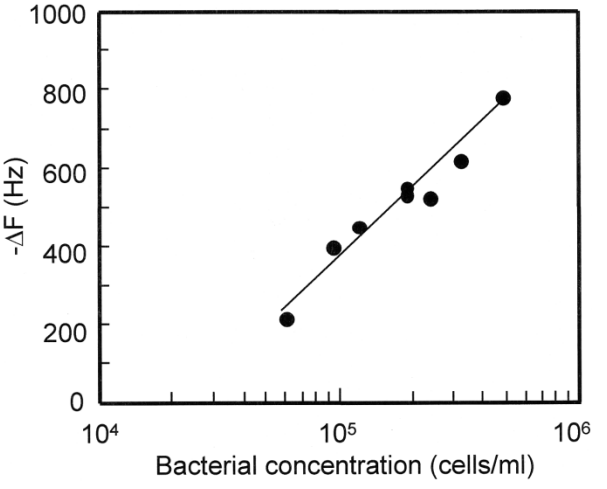

Figure 6. Correlation between the concentrations of nitrifying bacterial cells and variations in the resonant frequency (ΔF).

3.3 *Relationships between bacterial number and the resonant frequency of the QCA system*

An investigation was made on the relationships between the concentrations of the nitrifying bacterial cells and the variations in the resonant frequency ($-\Delta F$) (figure 6). The number of bacterial cells was measured using a microtiter technique according to the method of Yasuda *et al.* [1]. As shown in the figure, a good correlation was observed between the number of *N. europaea* cells and $-\Delta F$.

To discuss the validity of the frequency change, the weight of the latex beads in the chamber was calculated to be 0.55 mg according to the latex beads of 1 μm diameter, 100 μL, 10^{10} (particles mL^{-1}) and the relative density of 1.1. It is known that the 9 MHz quartz crystal has a sensitivity of 1 ng Hz^{-1}, the 0.55 mg corresponds to a 550 kHz frequency change. The frequency changes obtained in this experiment were much lower than the possible maximum value. This is explained by the fact that during the sedimentation of the latex beads the density of latex beads is not very different from that of water. Therefore, the sedimentation layer of the latex beads (layer A in figure 5) seems to be due to adsorption rather than deposition. One assay was completed within 10 min. Since the conventional microtiter technique requires 12–24 h of incubation, this system could be used for the rapid detection of nitrifying bacterial cells. Further studies in our laboratory are directed toward the application of this technique for the working site such as fish tanks equipped with biological filtration.

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