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# Use of Microelectrodes to Investigate the Effects of 2-chlorophenol on Microbial Activities in Biofilms

A short running title : Inhibition test using microelectrodes

By

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**Abstract**

In order to assess the applicability of using microelectrodes as a tool for inhibition tests, temporal and spatial inhibitory effects of 2-chlorophenol (2-CP) on $O_2$ respiration and nitrification activities in municipal wastewater biofilms were investigated using microelectrodes for $O_2$ and $NH_4^+$. The time-course microelectrode measurements demonstrated that 2-CP inhibited $O_2$ respiration and nitrification activities within 6 to 18 min. The microbial activities were inhibited only in the upper 400 μm of the biofilms by 2-CP, and the bacteria present in the deeper parts of the biofilms were still active, probably due to limited penetration of 2-CP. These results could reasonably explain the difference in inhibitory ratios of the $O_2$ respiration and nitrification activities in the biofilms. $O_2$ respiration activity was incompletely inhibited, which was attributed to the presence of $O_2$ respiration activities in the deeper parts of the biofilm. In contrast, nitrification activity was significantly inhibited because ammonia-oxidizing bacteria were present in the upper parts of the biofilm. These results indicate that the microelectrodes with a very quick response time and a high spatial resolution are useful tools to study temporal and spatial inhibitory effects of inhibitors on in situ microbial activities in biofilms.

**Keywords**

Inhibition of microbial activities; Biofilm; 2-chlorophenol; Microelectrodes

**Introduction**

It is well known that bacteria in biofilm are more resistant to inhibitors than are planktonic ones. It has been speculated that the reduced susceptibility of bacteria in biofilm is due to reduction of inhibitor penetration by the bacteria and extracellular polymeric substances, neutralization of inhibitors by biofilm constituents, and difference in the physiological state associated with lower growth rate (de Beer et al., 1994; Stewart et al., 2001; Stewart et al., 2000; Xu et al., 1996). For example, microelectrode measurements of chlorine concentrations in biofilms directly showed that chlorine concentrations in the

biofilms were typically only 20% or less of those in the bulk liquid (de Beer et al., 1994). Furthermore, microelectrode measurements of hydrogen peroxide penetration into biofilms revealed retarded penetration of the inhibitors (Stewart et al., 2000). Reduction of concentrations and hence the inhibitory effects of inhibitors in biofilm results in protecting bacteria in the deeper parts of the biofilm.

The inhibitory effects of inhibitors on microbial activities have been investigated using suspended cells (Blum and Speece, 1991; Hockenbury and Grady, 1977; Tomlinson et al., 1966). However, It is necessary to investigate the inhibitory effects on bacteria present in biofilm for the reason mentioned above. The inhibitory effects on bacteria in biofilm might be investigated by microelectrodes. The microelectrodes have been used for measurements of many reactants, intermediates and products of bacteria in biofilm (de Beer et al., 1997; Lorenzen et al., 1998; Okabe et al., 1999; Satoh et al., 2004). Therefore, application of the microelectrodes to the biofilm, which are exposed to inhibitors, makes it possible to investigate the inhibitory effects on microbial activities in the biofilm.

Industrial development in Japan has significantly grown, increasing the amount of synthesized organic compounds present in municipal and industrial wastewaters. Biological wastewater treatment processes using biofilms are widely used. Therefore, it is possible that shock loads of toxic compounds depress the microbial activities in the wastewater treatment processes. Decrease in wastewater treatment capacity leads to nuisance conditions, such as oxygen depletion and eutrophication of receiving waters. However, the inhibitory effects of inhibitors on the microbial activities in the wastewater biofilm have not been analyzed using microelectrodes.

In this study, we investigated temporal and spatial inhibitory effects of 2-chlorophenol (2-CP) on $O_2$ respiration and nitrification activities in municipal wastewater biofilms. We monitored $O_2$ and $NH_4^+$ concentration profiles in the municipal wastewater biofilms using microelectrodes. The total $O_2$ respiration and nitrification rates and the spatial distributions of these activities in the biofilms were calculated from these data. In addition, the spatial distributions of heterotrophic bacteria and ammonia-oxidizing bacteria were analyzed by fluorescence in situ hybridization (FISH) technique and were compared with the spatial distributions of their activities.

## Materials and Methods

### *Biofilm samples*

Biofilm samples were obtained from the primary aeration basin of a municipal wastewater treatment plant in Hachinohe, Japan (Satoh et al., 2003). The volume of the aeration basin was 322 $m^3$. The hydraulic retention time was approximately 10 h. The mixed liquor suspended solid (MLSS) concentration was about 2.8 g/L. The average $O_2$ concentration was 15 ± 10 µM (average ± standard deviation) in the aeration basin. Twenty acrylic plates (9.5 × 3.5 × 0.5 cm) were submerged in the aeration basin as a substratum for biofilms for microelectrode measurements and FISH analysis. After approximately three months, one acrylic plate covered with biofilm was taken from the aeration basin without disturbance and were transported to the laboratory.

### *Microelectrode measurements*

In the laboratory, concentration profiles of $O_2$ and $NH_4^+$ in the biofilms were recorded according to the protocol reported elsewhere (Satoh et al., 2004). Clark-type microelectrodes for $O_2$ with a tip diameter of approximately 15 µm and a 90% response time of <0.5 s were prepared and calibrated as described by Revsbech (1989). LIX-type microelectrodes for $NH_4^+$ (de Beer et al., 1997) were constructed, calibrated, and used according to a protocol reported elsewhere (Okabe et al., 1999). All measurements were performed in a flow cell (4.0 L) that was filled with an artificial medium at 20˚C. The medium was circulated at an average liquid velocity of 2 cm/s. The artificial medium used to monitor the concentration profiles consisted of $NH_4Cl$ (300 µM), $NaNO_3$ (300 µM), $Na_2HPO_4$ (570 µM), $MgCl_2•6H_2O$ (84 µM), $CaCl_2$ (200 µM), and EDTA•2Na (270 µM). The $O_2$ concentration and pH were kept at approximately 180 µM and 7.5, respectively. An acrylic plate covered with biofilm was placed in the flow cell, and to ensure that steady-state profiles were obtained, the biofilm was then acclimated in the medium for at least two hours before measurement. The concentration profiles in the biofilm were monitored by motor-driven micromanipulators (model MM-60V-H1 and MM-60XY-H1; Chuo Precision

Industrial Co., Ltd., Tokyo, Japan) at an interval of 100 µm from the bulk liquid into the biofilm. The biofilm surface was evaluated using a dissecting microscope (model Stemi 2000; Carl Zeiss).

After acclimation of the biofilm, three concentration profiles were measured at different positions in the biofilm for each species and set of conditions. Thereafter, 2-CP (Wako Pure Chemical Industries, Osaka, Japan) was added to the bulk liquid, resulting in final concentration of about 10 mg/L. 2-CP is common pollutants found in aquatic environments (Doong et al., 2002) and are often used as chemicals for inhibition tests (Blum and Speece, 1991). The concentration profiles of $O_2$ and $NH_4^+$ in the biofilms were monitored at regular time intervals during 45 min after addition of 2-CP. Each $O_2$ concentration profile could be recorded within approximately 5 min and each concentration profile of $NH_4^+$ could be recorded within approximately 15 min. Each time of the points in the figures indicates the point at which each microelectrode measurement was completed.

*Calculation of metabolic rates*

Based on the concentration profiles measured, net specific $O_2$ respiration and $NH_4^+$ consumption (i.e. nitrification) rates in the biofilms were calculated using Fick's second law of diffusion including a consumption term:

$$\partial C(z,t)/\partial t = D \times \partial^2 C(z,t)/\partial z^2 - R(z)$$

where C(z,t) is the concentration at time t and depth z, D is the molecular diffusion coefficient in the biofilm, R(z) is the net specific metabolic rate at depth z. After integration of this equation, we have $C_{n-1} = C_n + h \times (dC/dz_{n-1} + h \times A_{n-1})$, where Cn is the concentration measured with a microelectrode at time n, h is the step size of microelectrode measurement and $A_n = R_n / D$. Using this equation, we can calculate concentration profiles by altering the net specific metabolic rates and minimizing the sum of squared deviations of the calculated profile from the measured profile. We chose to use Microsoft EXCEL Solver to achieve this goal. The details of this method have been described previously by Lorenzen et al. (1998).

The total metabolic rate (J(µmol/cm$^2$/h)) of the biofilm was calculated using Fick's first law of diffusion:

$J = - D (dC/dz)$

where dC/dz is the measured concentration gradient of each solute in the boundary layer at the biofilm-liquid interface, and D is the molecular diffusion coefficient. Inhibition ratios of $O_2$ respiration and nitrification activities in the biofilms were calculated from the following equation:

$I = 1 - J_T / J_U$

where I is the inhibition ratio of the microbial activity in the biofilm, $J_T$ is the total metabolic rate of the biofilm treated with the inhibitor, and $J_U$ is the total metabolic rate of the untreated biofilm. The molecular diffusion coefficients used for the calculations were $2.09\times10^{-5}$ $cm^2/s$ for $O_2$ and $1.38 \times 10^{-5}$ $cm^2/s$ for $NH_4^+$ at 20˚C (Andrussow, 1969).

*FISH*

Biofilm samples taken from the aeration basin were fixed in 4% paraformaldehyde solution (Amann, 1995) and embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Vertical thin sections (20 µm thick) of the fixed biofilm were prepared after the biofilm had been frozen at –20˚C overnight. Dehydration and in situ hybridization were performed according to the procedure described by Amann (1995). The following oligonucleotide probes were used: EUB338 (specific for general bacteria; Amann et al., 1990), Nso190 (specific for ammonia-oxidizing bacteria belonging to the β subclass for the proteobacteria; Mobarry et al., 1996). All probes were synthesized and labeled at the 5’ end with tetramethylrhodamine 5-isothiocyanate (TRITC) or with fluorescein isothiocyanate (FITC) (TaKaRa Shuzo, Shiga, Japan). The previously published optimal hybridization conditions were used for each probe (Okabe et al., 1999). After hybridization and washing steps, the slides were allowed to air-dry, then mounted in antifading solution (Slow Fade Light; Molecular Probes, Eugene, Ore.). A confocal laser scanning microscope (model LSM 510, Zeiss) equipped with an Ar ion laser (488 nm) and a HeNe laser (543 nm) was used to detect and record probe-stained cells. All image combining, processing, and analysis were performed with the standard software package provided by Zeiss.

*Analytical methods*

2-CP concentrations were determined using a gas chromatograph (GC) (GC-17, Simadzu, Kyoto, Japan) equipped with a capillary column (DB-5MS, 30 m × 0.25 mm × 0.25 µm, J&W Scientific) and a mass spectrometry (MS) (QP-5050A, Simadzu). In preparation for extraction, each Sep-Pak PS-2 cartridge (Waters) was conditioned with 5 mL dichloromethane, 5 mL methanol and 5 mL pure water on a Solid Phase Extraction (SPE) manifold (Valian). Samples were then applied to Shimadzu SPE apparatus and were applied to PS-2 cartridge via a Teflon tube and adaptors. In SPE procedure, a flow rate of 20 mL/min was maintained. After extraction, PS-2 cartridge was dried in a vacuum of maximum value (drying time 60min). 2-CP was eluted from Sep-Pak PS-2 cartridge with 10 mL dichloromethane. The extract was evaporated to 0.3mL in centrifuge tube. Extract samples were reacted with 100 µL *N,O*-Bis(trimethylsilyl)trifluoroacetamide (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 60 min. The samples were filled up to 1mL with dichloromethane and analyzed by GC/MS.

The $NH_4^+$ concentration was determined colorimetrically (APHA, 1998). The samples were filtrated with 0.2 µm membrane filters before analysis. The $O_2$ concentration and pH in the medium for microelectrode measurements were continuously monitored using an $O_2$ electrode and a pH electrode, respectively.

**Results**

Average $O_2$ concentration profiles in the biofilm untreated and treated with 2-CP are shown in Fig. 1A. The concentration profiles were monitored 3 times in the untreated biofilm and 5 times in the treated biofilm during 33 min. The results showed a deeper penetration of $O_2$ in the treated biofilm than in the untreated biofilm. To investigate the spatial inhibitory effect of 2-CP on $O_2$ respiration activity in the biofilm, net specific $O_2$ respiration rates ($R(O_2)$) were calculated and their spatial distributions are shown in Fig. 1B. $O_2$ respiration activity was found at the biofilm surface to a depth of 600 µm with the maximum rate of 7.8 µmol/cm$^3$/h at a depth of 200 µm in the untreated biofilm. After addition of 2-CP, the rates decreased in the upper 400 µm of the biofilm, while the rates

increased below a depth of 600 μm in the biofilm. Therefore, $O_2$ respiration activity shifted to the deeper parts of the biofilm with addition of 2-CP due to deeper penetration of $O_2$. Changes in the total $O_2$ respiration rate ($J(O_2)$) and the inhibition ratio of $O_2$ respiration activity in the biofilm after addition of 2-CP are shown in Fig. 1C. The average total $O_2$ respiration rate (n=3) is indicated only in the untreated biofilm. Each time of the points in the Fig. 1C indicates the point at which each microelectrode measurement was completed. The total $O_2$ respiration activity was inhibited by 58% at 6 min after addition of 2-CP, and the inhibition ratio gradually decreased to 50% at 33 min.

Average $NH_4^+$ concentration profiles in the biofilm untreated and treated with 2-CP and a spatial distribution of net specific nitrification rates ($R(NH_4^+)$) in the untreated biofilm are shown in Fig. 2A and 2B, respectively. Nitrification occurred in the upper 400 μm of the untreated biofilm. In contrast, $NH_4^+$ concentration did not decrease in the treated biofilm (Fig. 2A). This result indicates that the nitrification activity was very low, which was attributed to inhibition of nitrification by addition of 2-CP. $NH_4^+$ concentration increased toward the bottom of the treated biofilm, probably due to biomass degradation and the liberation of $NH_4^+$ adsorbed on biomass in the biofilm. Changes in the total nitrification rate ($J(NH_4^+)$) and the inhibition ratio of nitrification activity in the biofilm after addition of 2-CP are shown in Fig. 2C.

During the microelectrode measurements, the 2-CP concentration in the bulk was measured regularly by GC/MS analysis (Fig. 3). The average 2-CP concentration was 10.7 ± 0.4 mg/L (average ± standard deviation, n = 4) at 0 min and it was almost unchanged during the experiments. This result indicates that loss of 2-CP during the experiments by volatilization, degradation by bacteria, and adsorption on the biofilm surface and experimental equipment had an insignificant effect on the bulk 2-CP concentration.

The in situ spatial distributions of heterotrophic bacteria and ammonia-oxidizing bacteria within the biofilm were visualized by FISH analysis (Fig. 4). The microscopic image of vertical cross sections of the biofilm revealed that the biofilm was densely packed and the biofilm surface was rough. Ammonia-oxidizing bacteria were present in the upper 400 μm of the biofilm and their population was higher in the upper 200 μm of the biofilm. In contrast, heterotrophic bacteria (including ammonia-oxidizing bacteria) stained with general bacterial probe EUB338 were detected at the biofilm surface to a depth of 1000 μm

although their population was very low below a depth of 600 μm in the biofilm.

## Discussion

Microelectrode measurements could clearly reveal the location of microbial activities in the biofilms disturbed by 2-CP with a high spatial resolution. The spatial distributions of net specific $O_2$ respiration and nitrification rates in the treated biofilms indicated that the microbial activities were inhibited only in the upper 400 μm of the biofilms and the bacteria present in the deeper parts of the biofilms were still active. These results could be explained by limited penetration of 2-CP. Based on these results, we could speculate that 2-CP penetration depth was about 400 μm in the treated biofilm during the experiments. Limited penetration of inhibitors into biofilms has been widely observed in previous investigations (de Beer et al., 1994; Stewart et al., 2001; Stewart et al., 2000; Xu et al., 1996). In those reports, the concentration profiles of hydrogen peroxide or chlorine in biofilms were directly measured with microelectrodes and limited penetration of the biocides into the biofilms was demonstrated. This phenomenon could be explained by several mechanisms: diffusional resistance of the biofilm matrix (Costerton et al., 1987), neutralizing reaction of the inhibitors with the biofilm constituents (van der Wande et al., 1989), adsorption of the inhibitors onto the biofilm constituents, and degradation of the inhibitors (Antizar-Ladislao and Galil, 2003).

The difference between the spatial distributions of heterotrophic bacteria and ammonia-oxidizing bacteria in the biofilm could explain the difference between the inhibition ratios of their activities. $O_2$ respiration activity in the biofilm was incompletely inhibited by 2-CP (Fig.1B), which was attributed to the presence of active heterotrophic bacteria in the deeper parts of the biofilm, where the inhibitor concentration might be low. In contrast, nitrification activity was significantly inhibited by 2-CP, because ammonia-oxidizing bacteria were present mainly in the upper parts of the biofilm. Moreover, it is well known that nitrifying bacteria were more susceptible to many types of the inhibitors than heterotrophic bacteria (Blum and Speece, 1991).

Wastewater treatment plants frequently fail to establish stable performance due to toxic loads in the influent (Jonsson et al., 2000), thus engineers involved with wastewater

treatment plant operation may need to know how quickly microorganisms respond to inhibitors. In this study, the time series of total metabolic rates (J) in the biofilms showed immediate inhibition of $O_2$ respiration and nitrification activities by 2-CP. Thus, the use of the microelectrodes with a very quick response time made it possible to investigate the short-term impact (a few minutes) of 2-CP on the microbial activities in the biofilms. In contrast, the traditional cultivation-based methods required a relatively long time (from a few hours to days) to analyze the inhibitory effects (Blum and Speece, 1991). Consequently, we suggest that microelectrodes are useful tools to study the short-term inhibitory effects of inhibitors on microbial activities in biofilms.

## Conclusions

$O_2$ respiration and nitrification activities in the biofilms treated with 2-CP were analyzed by the use of microelectrodes to investigate the inhibitory effects of 2-CP on the microbial activities. Microelectrodes with a very quick response time and a high spatial resolution were useful tools to study the mechanisms of inhibition of the microbial activities in the biofilm. The time-course microelectrode measurements demonstrated that 2-CP inhibited $O_2$ respiration and nitrification activities within 6 to 18 min. The microbial activities were inhibited only in the upper parts of the biofilms, whereas the bacteria present in the deeper parts of the biofilms were still active. This appeared to be attributed to the limited penetration of 2-CP into the biofilms.

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**List of Figures**

**Fig. 1.** The average $O_2$ concentration profiles (A) and the spatial distributions of net specific $O_2$ respiration rates (B) in the biofilm untreated and treated with 2-CP. Time-dependent changes in the total $O_2$ respiration rate ($J(O_2)$) and the inhibition ratio of $O_2$ respiration activity after addition of 2-CP (C). Error bars represent standard deviations. Zero on the horizontal axis corresponds to the biofilm surface.

**Fig. 2.** The average $NH_4^+$ concentration profiles in the biofilm untreated and treated with 2-CP (A) and the spatial distributions of net specific nitrification rates in the untreated biofilm (B). Time-dependent changes in the total nitrification rate ($J(NH_4^+)$) and the inhibition ratio of nitrification activity after addition of 2-CP (C). Error bars represent standard deviations. Zero on the horizontal axis corresponds to the biofilm surface.

**Fig. 3.** Changes in 2-CP concentration in the bulk during the microelectrode measurements. Data are means of four measurements and error bars represent their standard deviations.

**Fig. 4.** Confocal laser scanning microscope images showing the in situ spatial distridutions of nitrifying bacteria and heterotrophic bacteria in the municipal wastewater biofilm. (A) FISH with TRITC-labeled probe Nso190. (B) FISH with FITC-labeled probe EUB338. Bar = 200 μm. The biofilm surface is the top of the picture.

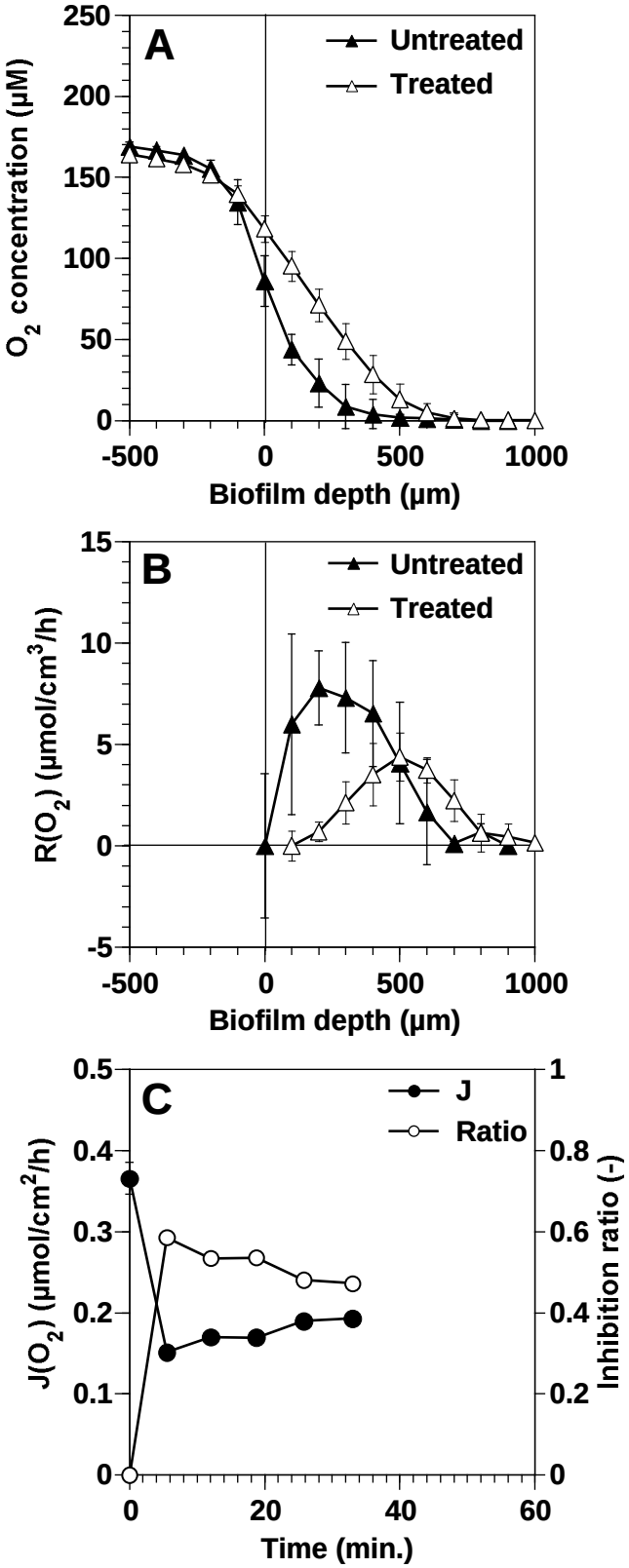


**Fig. 1**
**Use of microelectrodes to investigate the effects of 2-chlorophenol on microbial activities in biofilms.**
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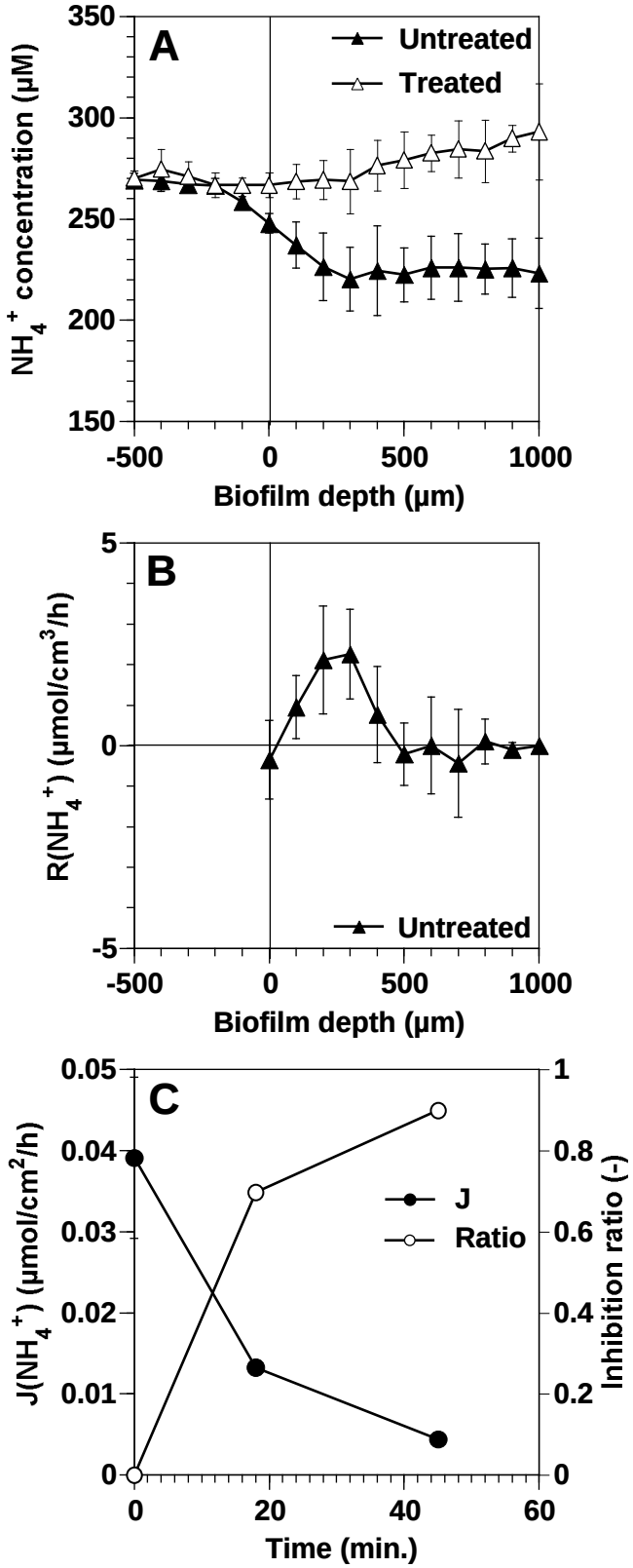


**Fig. 2**
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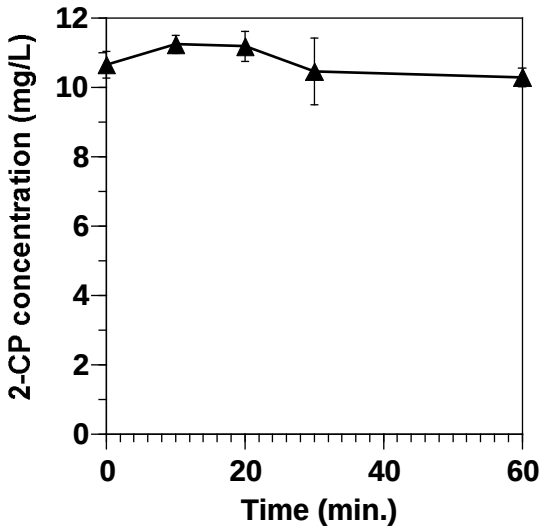


**Fig. 3**
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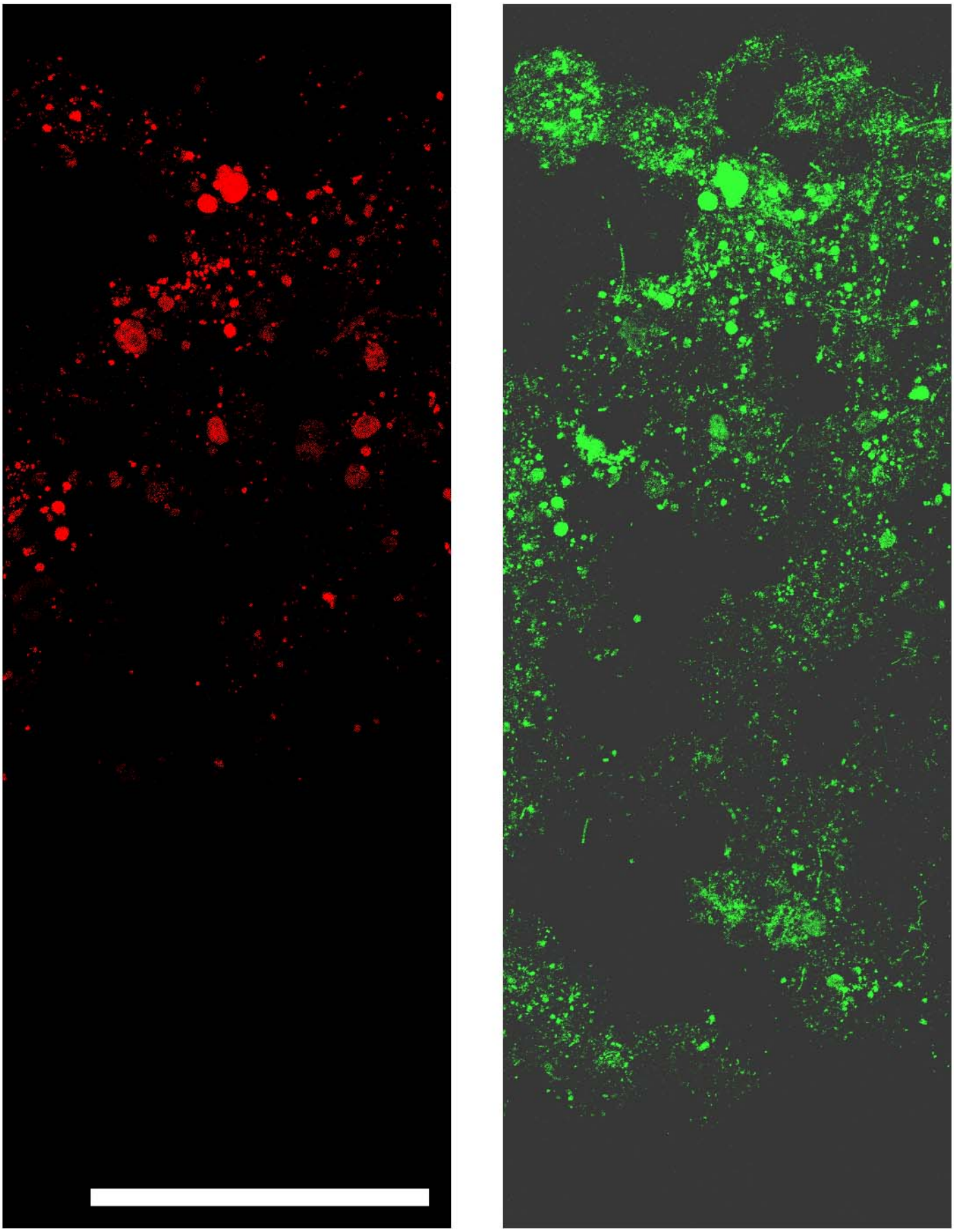

Fig. 4
Use of microelectrodes to investigate the effects of 2-chlorophenol on microbial activities in biofilms.
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