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Vertical profiles of current velocity and dissolved oxygen saturation in biofilms on artificial and natural substrates

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Abstract We determined vertical changes in current velocity and dissolved oxygen concentration in biofilms on artificial and natural substrates using microelectrodes. We used biofilms developed on glass slides dipped in an artificial stream for 3 months, artificial clay tiles dipped in an outdoor artificial stream for 3 months, and natural pebbles. In the biofilm on a glass slide, current velocity significantly decreased from the surface of the biofilm and became 0 cm s^{-1} at the surface of the glass slide. Vertical profile of current velocity versus depth indicated a presence of a viscous sublayer of 0.2-mm thickness above the surface of glass slide. Dissolved oxygen (DO) concentration increased within the biofilm and attained the maximum (123%) at the surface of the glass slide, indicating active photosynthesis by sessile diatoms at the layer corresponding to the observed viscous sublayer. In the biofilm on an artificial tile, DO increased to 163% saturation at 24.849-mm depth, followed by rapid decrease (6%) at the surface of the tile. A similar result of remarkable decrease in DO saturation was also found in the biofilm on a natural pebble. These results suggest that smoothness of the substrate surface is related to the vertical profile of DO saturation. The thickness of the viscous sublayer and oxygen-depleted area (up to several hundred micrometers, μm) was sufficient for the presence of bacteria, protists, and other metazoan animals, suggesting high activity and diversity of those heterotrophs in the bottom part of biofilms.

Key words Biofilms · Stream pebbles · Microelectrode · Vertical profile · Dissolved oxygen · Current velocity

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Introduction

Flows in aquatic environments produce shearing stresses on the streambed. Thus, stream environments may be considered to be inadequate habitats for loosely attached organisms. However, current velocity decreases near the substratum following logarithmic law, and it finally becomes negligible or zero in a thin layer adjacent to the substratum surface, which is the so-called viscous sublayer (Vogel 1994; Allan 1995). Because of the reduced current velocity, loosely attached microorganisms are able to settle, grow, and often develop a dense biofilm in this aquatic habitat (Silvester and Sleigh 1985; Fukuda et al. 2004). These biofilms, plus the great abundance of weakly attached protists and planktonic organisms on pebble biofilms in streams, make it likely that active microbial food webs exist there (Fukuda et al. 2004). Thus, the presence of the viscous sublayer is important for the ecology of stream microorganisms.

Microorganisms in biofilms are mainly responsible for autotrophic and heterotrophic processes in streambed environments (Stevenson 1996; Ward and Johnson 1996). Hence, determining physicochemical variations in or adjacent to the viscous sublayer in biofilms should be of primary importance for the elucidation of microbial processes in a stream ecosystem. As far as we know, however, these variations in biofilm have not yet been directly assessed, probably because of the technical difficulties in measuring current velocities in microscale resolution. Recently, some microelectrodes that can determine certain physicochemical variations such as current velocity, dissolved oxygen, and nitrate with microscale resolution have been developed (Revsbech and Jørgensen 1986; Revsbech 1989; Okabe et al. 1999; Ito et al. 2002). In aquatic environments, oxygen concentration is one of the most important physicochemical variations, because most organisms require oxygen for their metabolic activities, despite oxygen being in limited supply. Unfortunately, we still have limited information about oxygen concentration in or over biofilms in a stream, although oxygen availability may drastically change on a microscale

in those microhabitats. To our knowledge, only the work of Glud et al. (1992) is available on oxygen concentration in or over biofilms in a stream with microscale resolution. In this article, we present the results of the first trial to determine the vertical profile of dissolved oxygen concentration, together with that of current velocity, associated with biofilms on artificial and natural substrates using the respective microelectrodes.

Materials and methods

We used three biofilms developed on the following materials for the experiments: glass slides dipped in the artificial stream in the laboratory in Ehime University Forest, artificial tiles dipped in the outdoor-artificial streams in Lake Biwa Museum, and natural pebbles collected from Ishite Stream, Matsuyama, Ehime, Japan, in September 2003. The thickness of the biofilm on the natural pebble was 0.5–1 mm.

The artificial stream at the laboratory in Ehime University Forest was designed using a polyvinyl chloride pipe cut in half (width × height × length = 10 cm × 7 cm × 200 cm) and filled with water collected from Ishite Stream (Fukuda et al. 2004). The stream water was circulated using a pump (model SL-3S; Elepon, Tokyo, Japan), and we added NH_4NO_3, KH_2PO_4, and $NaSiO_3 \cdot 9H_2O$ to the artificial stream every 2 weeks at the final concentrations of 100 µmol N l^{-1}, 5 µmol P l^{-1}, and 100 µmol Si l^{-1}, respectively. Glass slides were submerged in the artificial stream between July and September 2003 and allowed to develop biofilms on the surfaces, and we had biofilms 0.5–1 mm thick. We also used the outdoor-artificial stream of Lake Biwa Museum to develop biofilms. The outdoor-artificial stream was designed using a Fiber Reinforced Plastic (FRP) pipe (length × width × depth = 30 m × 0.2 m × 0.15 m). The pipe was inclined, and the higher end of the pipe was continuously supplied with water pumped from Lake Biwa. In this experiment, artificial clay tiles (length × width × depth = 7.0 cm × 2.0 cm × 2.0 cm; INAX, Tokoname, Japan) were dipped between September and November 2003 to develop biofilms. The thickness of the biofilm was about 2 mm.

To determine vertical profiles of current velocity and dissolved oxygen (DO) concentration, we used Microsensors (Unisense, Aarhus, Denmark) as microelectrodes. A flow microsensor (FS-20; Unisense) with a 20-µm tip diameter and an oxygen microsensor (OX-10; Unisense) with a 8- to 10-µm tip diameter were used for the respective measurements in the biofilms on the glass slides, artificial clay tiles, and natural pebbles. For current velocity and DO measurements, we followed the methods of Vopel et al. (2002) and Revsbech (1989), respectively. Calibration of the flow microsensor was conducted in a half-cut aluminum pipe (diameter = 1 cm) placed in the artificial stream system to minimize turbulent flow caused by subtle wind effects. We could not obtain successful results in the artificial stream. In our laboratory, there were subtle wind effects that we could not detect due to negligible wind velocity in a random direction, and it is likely that these caused turbulent flow in the artificial stream. For this reason, we used a thin aluminum half-pipe. We changed current velocities between 0 and 10 cm s^{-1} using a pump (model SL-3S; Elepon). A piece of styrofoam (1 mm × 1 mm × 1 mm) was put on the surface water running in the aluminum half-pipe, and we determined the time for the piece to move a certain distance. Simultaneously, we dipped the microelectrode tip into the surface water of the aluminum half-pipe, and the data were acquired by a picoammeter (PA2000; Unisense), logging continuously with a frequency of 50 Hz. The electric current from the microelectrode was amplified and converted by the picoammeter, and the data were digitized by an analogue-to-digital converter. Then, these data were stored in a PC using Profix version 2.2 software (Unisense). The result of our calibration of current velocity using an aluminum half-pipe is shown in Fig. 1. Using the relationship in Fig. 1, we converted the electric voltage data into current velocity.

For calibration of oxygen microsensors, we prepared aerated distilled water by vigorous bubbling for longer than 5 min and a strong reductant solution by dissolving sodium ascorbate and NaOH in distilled water at a concentration of 0.1 mol l^{-1}. We kept the microelectrode tip in either of the two liquids, one of oxygen saturation and the other of oxygen depletion, read their electric signals as described above, and calibrated the microelectrodes. Our DO measurements were conducted under a fluorescent lamp for biofilms developed on a glass slide, a natural pebble, and an artificial tile in an outdoor-artificial stream. The coefficient of variation of DO measurement using microsensors in the present study was 1.1%. We analyzed DO profiles thus measured using the numerical model Profile version 1.0 (Berg et al. 1998). The model calculates the net consumption rate as a function of depth on the assumption that the concentration–

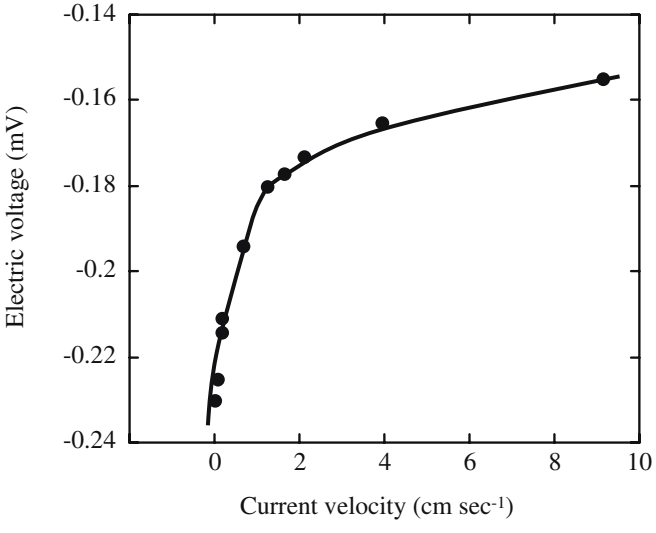

Fig. 1. Relationship between current velocity and electric voltage derived from microelectrode measurement of the velocity. Because a relationship between the two variables does not follow any fixed equation (Unisense A/S, personal communication), we fitted the measurements by eye following the suggestion by the company

depth profiles are in a steady state. The model is based on a series of least-square fits to measured concentration profiles, assuming an increasing number of production and consumption zones, and followed by comparisons of these fits through statistical *F* testing. The measured steady-state DO profiles were analyzed with the model by assuming a constant porosity of 0.9 (Jørgensen and Cohen 1977; Jørgensen et al. 1979; Wieland and Kühl 2000a,b; Wieland et al. 2001) and a constant D_s, which was calculated as follows (Ullman and Aller 1982; Wieland et al. 2001):

$$D_s=\varphi^2 D_0$$

where D_0 is the free solution molecular diffusion coefficient of O_2 (Broecker and Peng 1974) and φ is the porosity.

To estimate algal species composition (including cyanobacteria), we collected the biofilms from the tiles and the pebbles by brushing, after determining velocity and DO. Each biofilm sample was suspended in distilled water and immediately fixed with 1% formaldehyde. In the laboratory, each biofilm sample was either concentrated or diluted to an extent that the biofilm from 100 mm^2 of the substratum was suspended in 1 ml 1% formaldehyde solution. A part of the sample was then placed on an optical plastic plankton counter (Matsunami Glass, Kishiwada, Japan) and examined using an inverted microscope IX70 (Olympus, Tokyo, Japan) at a magnification of ×400. Cell or trichome den-sities of each algal genus on the substratum were estimated.

Results and discussion

In the experiment using biofilm on a glass slide, we measured the current velocity at 1- or 2-mm intervals from depths at 0.000 to 35.100 mm and at 50- or 100-µm intervals from depth at 35.100 mm to the surface of the glass slide, respectively. Current velocity significantly decreased from 0.000 mm (Fig. 2A), and was almost 0 cm s^{-1} at the surface of the glass slide (Fig. 2B). Current velocities between depth at 36.750 mm and the surface of the glass slide showed linear changes (Fig. 2B; r^2 = 1.000). The profile shown in Fig. 2 indicates the presence of the viscous sublayer within 0.2 mm above the surface of the glass slide (Dade et al. 2001).

For measuring DO profile in the biofilm on a glass slide, we vertically scanned the microelectrode at 1-mm intervals from 0.000 to 30.000 mm, at 0.5-mm intervals from depth 30.500 to 32.500 mm, at 0.25-mm intervals from depth 32.750 to 33.250 mm, at 100-µm intervals from 33.350 to 33.750 mm, at 50-µm intervals from 33.800 to 33.850 mm, at 10-µm intervals from 33.860 to 33.910 mm, and at 5-µm intervals from 33.915 mm to the surface of the glass slide (Fig. 3). DO saturation ranged between 97.2% and 98.7% from 0.000 to 33.750 mm (Fig. 3A) and increased from depth 33.800 mm, attaining the maximum (123%) at the surface (Fig. 3B). Thus, the thickness of the DO supersaturation layer (Fig. 3B) approximately corresponded to the thickness of the viscous sublayer (see Fig. 2B). The presence of the thin DO supersaturation layer suggested active photosynthesis by sessile algae, although we did not identify the dominant algal species.

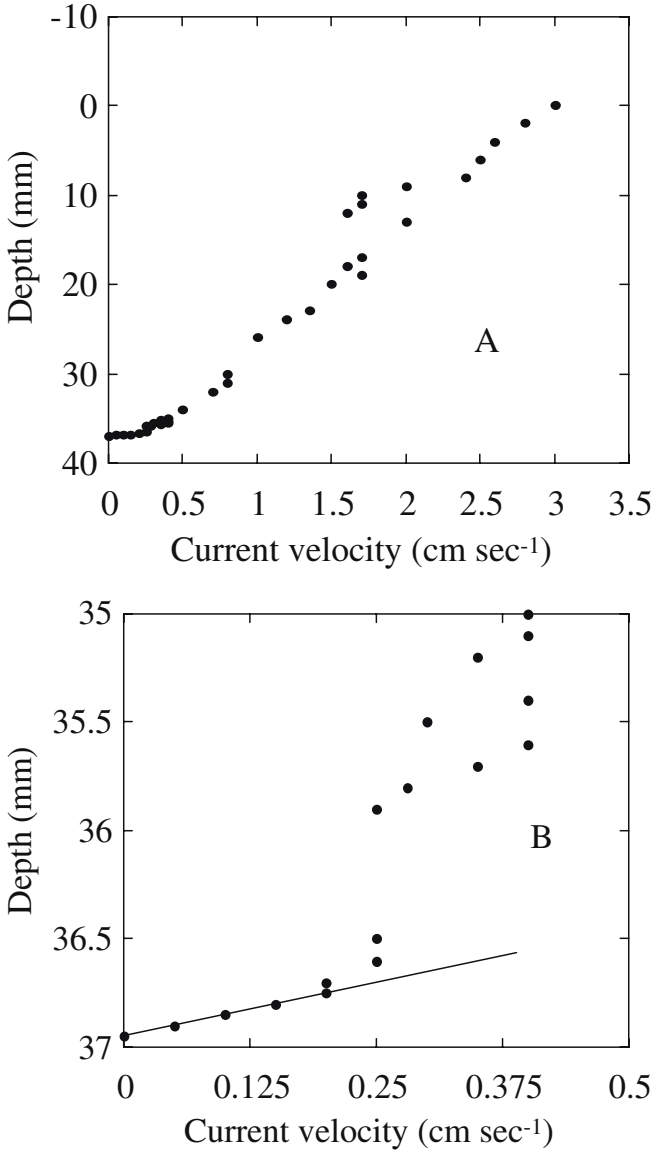

Fig. 2A,B. Vertical profile of current velocity in the experiment conducted at the Forest Research Center using biofilm on a glass slide as substrate. **A** Vertical profile within 36.950-mm thickness from the surface of a glass slide; **B** enlarged profile from depth at 35.000 mm to the surface of a glass slide. The depth of the interface between water and biofilm was probably about 36.000 mm

Vertical profiles of DO saturation in biofilms developed on an artificial tile and a natural pebble were different from that of a glass slide (Fig. 4). In an experiment using an artificial tile, DO saturation increased from 122% at 23.298 mm to 163% at 24.849 mm but decreased remarkably below 24.855 mm and attained 6% at the surface of the tile (Fig. 4A). This DO profile suggests that the photosynthetically active biofilm on the tile (Fig. 4A) was thicker than that on the glass slide (Fig. 3). The biofilm was mainly composed of *Leptolyngbya* (Cyanoprocaryota) and *Cosmarium* (Charophyceae). *Leptolyngbya* forms free

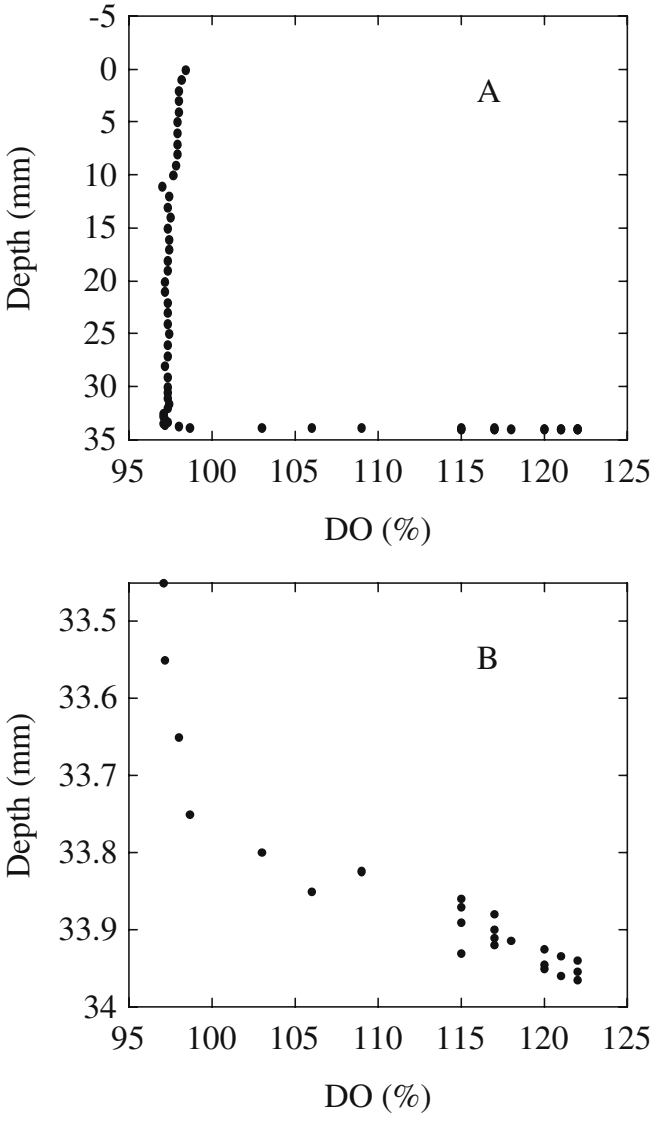

Fig. 3A,B. Vertical profiles of dissolved oxygen (*DO*) in the biofilm on a glass slide as substrate. **A** Vertical profile within 33.965-mm thickness from the surface of a glass slide; **B** enlarged profile from depth 33.450 mm to the surface of a glass slide. The depth of the interface between water and biofilm was probably about 33.500 mm

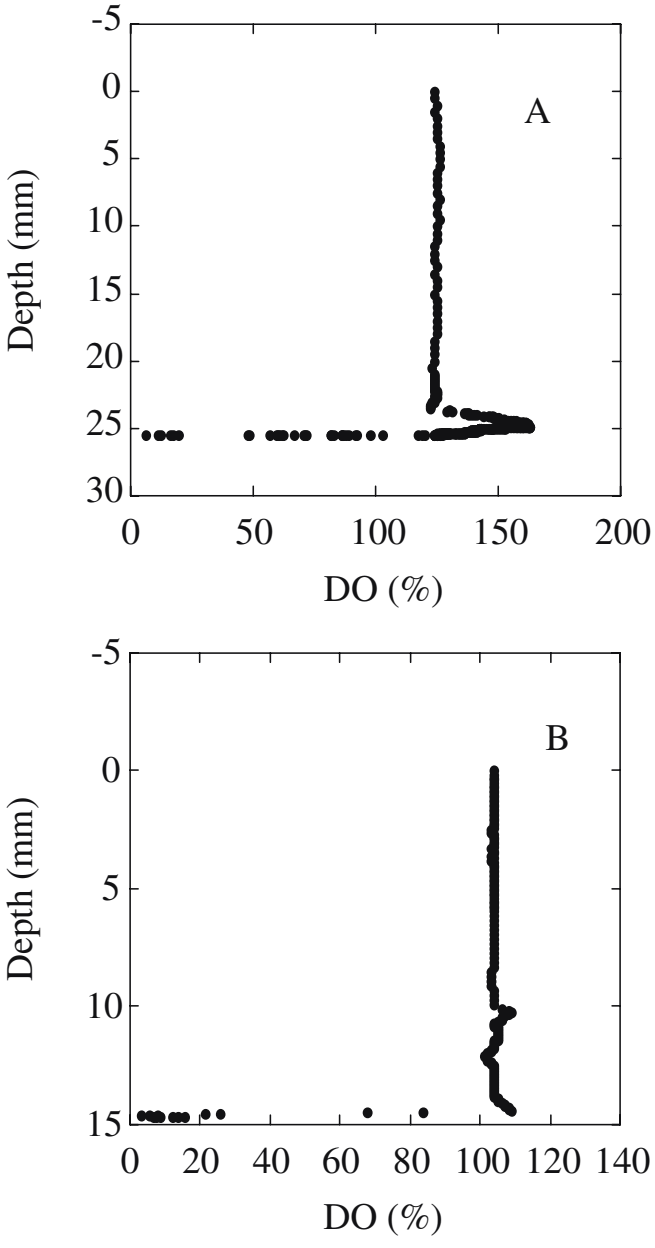

Fig. 4. Vertical profiles of *DO* in biofilms on an artificial tile (**A**) and a natural pebble (**B**) as substrates; the depths of the interface between water and biofilm were probably about 23.600 mm and 14.000 mm, respectively

clusters of tangled trichomes without adhesive apparatus (Anagnostidis and Komárek 1988). *Cosmarium* generally lives solitarily and has a motile life form (Hoek et al. 1995). Although their adhesions to the substratum are not considered strong, they could develop a relatively thick biofilm in the outdoor-artificial stream of Lake Biwa Museum with water velocity less than 10 cm s^{-1}. Similar results of remarkable decrease in DO were detected in an experiment using biofilm on a natural pebble, although no distinct increase in supersaturation in oxygen was found (Fig. 4B).

The interpretation of measured DO profiles in Figs. 3 and 4 is shown in Figs. 5, 6, and 7. The correlation between the measured and calculated DO profiles in a biofilm developed on a glass slide was insignificant (Fig. 5). Because there was an excess amount of data in Fig. 4A, we had to separately analyze the DO profile between the 24.809- and 24.861-mm depths and between the 25.475- and 25.507-mm depths (Fig. 6). The correlation between the measured and calculated DO profiles in the former part was insignificant (Fig. 6A), but we found a significant correlation in the latter part (Fig. 6B; $r^2 = 0.9326$, $P < 0.01$). On the natural pebble, we again found high correlation (Fig. 7; $r^2 = 0.9935$, $P < 0.01$) between the measured and calculated DO profiles. The patterns of O_2 production/consumption in Figs. 6B and 7 showed significant correlations between the measured and calculated DO profiles that agreed well with those of the measured and calculated DO profiles (Figs. 6B, 7). A fixed diffusivity in water (1.17×10^{-5} cm^2 s^{-1}; Manual for Profile version 1.0; Berg et al. 1998) and porosity (0.9) were used in

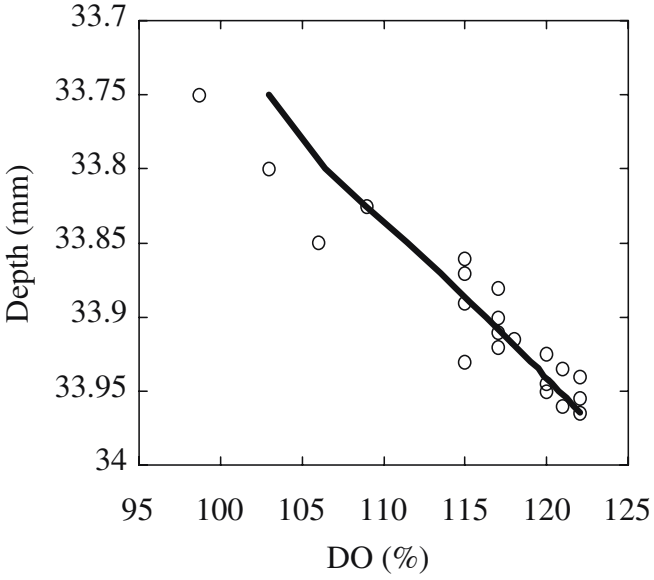

Fig. 5. DO profiles measured using a microelectrode (*circles*) and calculated using the software Profile version 1.0 (*thick line*) in biofilm developed on a glass slide

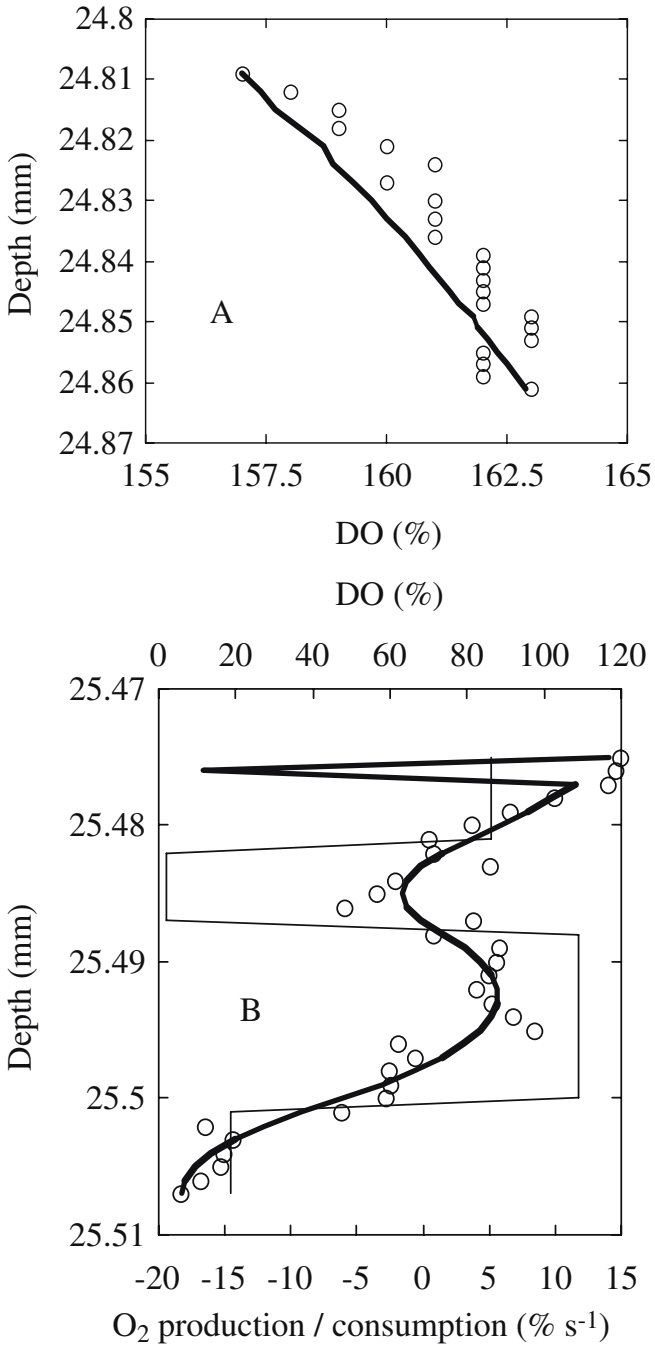

Fig. 6A,B. DO profiles measured using a microelectrode (*circles*) and calculated using the software Profile version 1.0 (*thick line*) in biofilm developed on an artificial tile. Because of an excess amount of data in Fig. 4A, we divided the profile into two parts: between 24.809- and 24.861-mm depths (**A**) and between 25.475- and 25.507-mm depths (**B**). For **B**, the calculated depth profile of O_2 production/consumption zones (*thin line*) is also shown

the present study, and the reason for the insignificant fits between the measured and calculated DO profiles in the biofilms of Fig. 5 and Fig. 6A might be the variable diffusivity and porosity in the biofilms.

The agreement between the O_2 production/consumption pattern and the measured and calculated DO profiles in Fig. 7 indicates a high flux of O_2 produced between 14.400 and 14.500 mm because of high D_s (see Materials and methods). Thus, the lack of a supersaturation layer was probably caused by the architecture of the biofilm. The dominant algal species in the biofilm were *Achnanthidium* (Bacillariophyceae), *Cocconeis* (Bacillariophyceae), and an unidentified coccoid cyanoprocaryote. They all were considered as taking an adnate life form, or forming thin and tightly piled-up aggregations (Tanaka and Watanabe 1990; Ohtsuka 1999). They might not form a mucilaginous layer on the pebble surface, and thus excess oxygen produced by photosynthesis could not remain near the surface for a long period. Also, the surface of the natural pebble was rough and hollow. Thus, the DO in a small hole would be consumed by a decrease in light intensity and high heterotrophic activities in such microstructures, and we could detect the decrease of oxygen concentration when the oxygen sensor reached the hole. This microhabitat may be the reason for the steep DO decrease profile shown in the biofilm on the artificial tile (Fig. 4A), although the surface of the artificial tile was smoother than that of the natural pebble.

The steep DO decrease near the substratum (see Fig. 4) may indicate the dominance of heterotrophic processes. It had been assumed by some researchers that such an environment should be anoxic because a biofilm usually contains many heterotrophic microorganisms and light may be deficient at the bottom of the biofilm (Sanders 1966; Lock et al. 1984). Biofilms consist of various microorganisms such as bacteria, cyanobacteria, algae, and fungi. These microorganisms develop a microbial exopolysaccharide matrix (Lock et al. 1984) that binds bacteria, microalgae, and fungi together, producing a complex microbial biofilm community with a tight internal cycling of nutrients between heterotrophs and autotrophs. Biofilms thus established have vertical profiles of physicochemical and biological

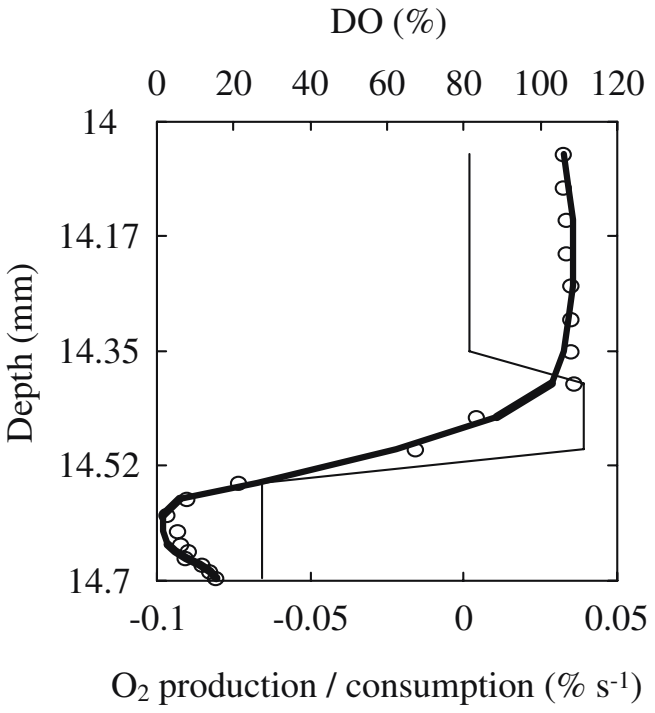

Fig. 7. DO profiles measured using a microelectrode (*circles*) and calculated using the software Profile version 1.0 (*thick line*) in biofilm developed on a natural pebble. Calculated depth profile of O_2 production/consumption zones (*thin line*) is also shown

variables. The thickness of the viscous sublayer and/or the oxygen-depleted area (up to several hundred micrometers; see Fig. 4) was enough for the presence of bacteria, protists, and other metazoan animals, suggesting high activity and diversity of those heterotrophs in the bottom part of biofilms. In addition, even under photic condition, other oxygen consumption processes such as Mehler reaction and photorespiration are possible (Wieland and Kühl 2000a,b), although we did not determine light irradiance in the present study.

Further studies are needed to elucidate the structure and function of biofilms in stream environments.

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