Rapid depletion of dissolved oxygen in 96 well microtitre plate *Staphylococcus epidermidis* biofilm assays promotes biofilm development and is influenced by inoculum cell concentration

Running title: Oxygen depletion in 96 well plate biofilm assays

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Abstract

Biofilm-related research using 96 well microtitre plates involves static incubation of plates indiscriminate of environmental conditions, making oxygen availability an important variable which has not been considered to date. By directly measuring dissolved oxygen concentration over time we report here that dissolved oxygen is rapidly consumed in *Staphylococcus epidermidis* biofilm cultures grown in 96 well plates irrespective of the oxygen concentration in the gaseous environment in which the plates are incubated. These data indicate that depletion of dissolved oxygen during growth of bacterial biofilm cultures in 96 well plates may significantly influence biofilm production. Furthermore higher inoculum cell concentrations are associated with more rapid consumption of dissolved oxygen and higher levels of *S. epidermidis* biofilm production. Our data reveal that oxygen depletion during bacterial growth in 96 well plates may significantly influence biofilm production and should be considered in the interpretation of experimental data using this biofilm model.

Introduction

96 well plates have mainly been applied to routine laboratory assays and high throughput drug discovery, many in automated processes (Sandberg *et al.*, 2008). Originally used as batch "mini-bioreactors" to maintain clonal libraries of facultative anaerobes such as *Escherichia coli* and yeasts, some recent studies have examined mixing and mass transfer of oxygen into the liquid media (Zhang *et al.*, 2008). Variations in shaking the plates and adjustments to improve mass transfer have been performed (Micheletti *et al.*, 2006), but plates are still often used statically in microbiological experiments, particularly for

biofilm assays. In the case of aerobic fermentations using fast growing cultures, oxygen quickly becomes rate limiting, because the solubility of oxygen in water/growth medium is low (Shin *et al.*, 1996).

Communities of bacteria that adhere to a surface and grow in a matrix-enclosed structure are known as biofilms (Characklis and Cooksey, 1983). Macro scale biofilm systems, such as glass capillary flow cells (Stoodley *et al.*, 2001), the rotating disk reactor (Zelver *et al.*, 1999) and the CDC reactor (Goeres *et al.*, 2005), are commonly used to study biofilm characteristics, such as structure and susceptibility to antibiotics (Stewart and Costerton, 2001).

Polystyrene 96-well plates have become one of the most popular methods for micro scale biofilm investigations (Christensen *et al.*, 1985) and are commonly used in biofilm genetic studies (Davey and O'Toole, 2000) to analyse differences in the quantity of biofilm formation in engineered mutants. A downside of this technique is the considerable experiment to experiment variation in biofilm production by individual bacterial strains. Nevertheless the technique is useful in determining whether bacterial strains are capable of forming biofilm (Peeters *et al.*, 2008). Specific modifications have been made to the basic 96 well plate method (Christensen *et al.*, 1985) by different groups, particularly in terms of the inoculum cell concentration and culture volumes. Table 1 indicates the range of inoculum cell concentrations and culture volumes used in *Staphylococcus epidermidis* 96 well plate biofilm assays. Biofilm experiments using 96 well plates are typically incubated at the optimal growth temperature without shaking and without consideration to the oxygen demand of the cells.

In vitro studies of staphylococcal biofilm development have revealed that anaerobic conditions promote production of the *icaADBC* operon encoded polysaccharide intercellular adhesin (PIA) (Heilmann *et al.*, 1996), an important component of the staphylococcal biofilm matrix, in both *S. aureus* and *S. epidermidis* (O'Gara, 2007). Oxygen limitation may therefore influence biofilm thickness. It can reasonably be assumed that in thicker biofilms, nutrient limitation is more likely at the substratum-biofilm interface, which in turn will influence biofilm physiology (Rani *et al.*, 2007). Slow-growing cells under nutrient-limited conditions have been shown to be more resistant to antibiotics (Xu *et al.*, 2000). Increased biofilm thickness also results in more locally anoxic regions or "pockets" forming within the deeper regions (Rani *et al.*, 2007). The objective of this work was to directly measure levels of dissolved oxygen available to *S. epidermidis* 1457 cells forming biofilm in 96 well plates, in order to determine if oxygen limitation influences the biofilm phenotype in these temperature and oxygen-controlled conditions.

Materials and Methods

Bacterial strain and media. *Staphylococcus epidermidis* 1457 (Mack *et al.*, 1992), a known strongly adherent biofilm producing strain, was stored in Protect beads at -80°C, and revived in brain heart infusion (BHI, Oxoid) broth overnight at 37°C.

Biofilm quantification in 96 well plates. Overnight cultures were adjusted to A_{660}=1.0 using sterile BHI to prepare a standard inoculum for biofilm assays. This standard inoculum equated to log 7.84 ± 0.01 colony forming units (CFU) ml^{-1}. Experiments were also undertaken using lower and higher inoculum cell concentrations. The "low" inoculum consisted of log 6.43 ± 0.02 CFU ml^{-1} and the "high" inoculum represented log

91 8.97 ± 0.01 CFU ml^{-1}. Both were created by dilution of the overnight culture with sterile
92 BHI, adjusted to different densities.
93 1 in 100 µl dilutions of the low, standard and high inoculums were added to each well in
94 the presence of the filtered gas in which the biofilm was to be cultured. 100 µl was used
95 in all wells for all reported experiments. Lids were placed on the plates before incubation
96 at 37°C, in a 10 l sterile sealed vessel, with a constant stream of filtered gas passed
97 through the headspace. The biofilm plates were incubated in an atmosphere of 100%
98 oxygen, or 21% oxygen / 79% nitrogen. All concentrations were verified off line prior to
99 operation using a gas analyser (Servomex 1400 gas analyzer, Sussex, U.K.). Evaporation
100 from the wells caused a negligible difference in liquid volume in the different oxygen
101 environments (data not shown).
102 Quantification of planktonic cells from 96-well plates was performed as described
103 previously, with 100 µl of the culture being mixed with 900 µl sterile Ringers solution
104 and serially diluted before being plated on BHI agar plates. To quantify the biofilm
105 adhering to the wells, the liquid culture was removed and the wells washed rigorously
106 three times with sterile Ringers solution to remove all planktonic cells. After the third
107 wash, 100 µl of Ringers solution were added to the wells and the plate was sonicated for
108 1 min to separate the biofilm from the base of the plate. The ultrasonic waves lifted the
109 biofilm from the plate into the Ringers, and this solution was further vortexed for 2 min
110 to ensure dispersal of any cell aggregates prior to performing serial dilutions and total
111 viable counts as described above.
112 **Dissolved oxygen measurements of planktonic cells**. For direct dissolved oxygen
113 readings, 96 well plate lids were pierced with a sterile 18G needle under aseptic

conditions and covered with UV sterilized parafilm. This enabled insertion of the needle-type oxygen microsensor (PreSens GmbH, Regensburg, Germany) into the centre of individual wells of a 96 well plate, 2mm from the top and bottom of the liquid, without removing the lid. From incubator to final measurement, the entire process took less than 2 min, which included the time allowed for the probe to stabilise and give a steady reading. Readings were the average of four measurements and were recorded every second. The dissolved oxygen in three wells from two plates was measured and the mean and standard error are presented.

Statistical analysis. Statistical analysis of dissolved oxygen, biofilm accumulation and total cell growth was performed by a two way analysis of variance (ANOVA).

Results

Dissolved oxygen is rapidly consumed by bacterial cultures in 96 well plate biofilm assays. Using a narrow tip oxygen sensor probe as part of a novel application, direct measurements of dissolved oxygen within an inoculated well of a 96 well plate were recorded. In Fig. 1A, *S. epidermidis* 1457 was cultured in a headspace atmosphere of air (21% oxygen). Complete depletion of dissolved oxygen was measured after 6 h. The same experiment performed in an atmosphere of 100% oxygen (Fig. 1B), also revealed that the dissolved oxygen concentration fell below the detectable level after 6 h but ultimately recovered to previously observed levels, and remained stable at ~ 7 mg L^{-1} after 24 h. As previously observed for this strain, less biofilm formed when oxygen was abundantly available (Cotter *et al.*, 2009).

Oxygen consumption promotes biofilm growth by *S. epidermidis* 1457 in 96 well microtitre plates. Profiles of dissolved oxygen concentrations and numbers of

138 planktonic cells at different oxygen concentrations are shown in Fig. 2. The total biofilm
139 formation of *S. epidermidis* 1457 in each well is shown as "Total biofilm CFU" in this
140 figure. The initial dissolved oxygen concentration is that of autoclaved BHI media and is
141 the same for all profiles. In an atmosphere of 0% or 21% oxygen, when dissolved
142 oxygen was consumed it remained below detection for the remainder of the experiment
143 (up to 24 h), though only the initial hours are shown in Fig. 2. A profile of dissolved
144 oxygen concentrations in 96 well plates incubated in an atmosphere of 100% oxygen
145 conditions is shown in Fig. 1B. The ratios of planktonic to biofilm cell counts in 96 well
146 plates incubated in an atmosphere of 0%, 21% or 100% oxygen are shown in Fig. 2D.
147 Predictably, at higher atmospheric concentrations of oxygen, the dissolved oxygen
148 concentrations in 96 well plates take longer to be depleted. Biofilm cell numbers were
149 statistically significantly higher under anaerobic conditions compared to 21% and 100%
150 oxygen conditions ($p<0.05$). Indeed, the ratio of planktonic to biofilm cell numbers
151 revealed that overall in the 96 well plate cultures a higher fraction of the biomass exists
152 as biofilm under anaerobic compared to aerobic conditions.
153
154 **Inoculum cell concentrations influence oxygen consumption and biofilm formation.**
155 Three different initial cell concentrations (low, standard and high inocula) of *S.*
156 *epidermidis* 1457 were used to inoculate 96 well plate cultures in an atmosphere of 100%
157 oxygen. Fig. 3 shows dissolved oxygen concentrations, biofilm cell numbers, total
158 bacterial cell numbers and the ratio of planktonic to biofilm cell numbers under these
159 conditions. The ratio of planktonic to biofilm cell numbers reaches 1.4 ± 0.15 after 4 h
160 irrespective of the inoculum cell concentration. The oxygen demand in the first few
161 hours of growth depends strongly on the cell concentration of the inoculum, as the

different biomass quantities are associated with different dissolved oxygen concentrations. The onset of oxygen limitation occurs at approximately 1, 3 and 6 h for the high, standard and low inoculum cell concentrations respectively. Statistical differences are observed from hours 1-4 in graphs A-C between the high inoculum and the other inocula ($p<0.05$). A difference is noted between the total bacterial growth in the standard and low inocula ($p<0.05$). The development of biofilm following inoculation with high, standard and low cell concentrations, arbitrarily defined as $> 10^4$ CFU cm^{-2}, occurs after 1 h, 1 h and 3 h, respectively (Fig. 3B), apparently reflecting the rate at which dissolved oxygen is consumed within these cultures.

Discussion

In this study we profiled dissolved oxygen concentrations during growth of bacterial biofilms in 96 well microtitre plates. Despite the pleiotrophic impact of oxygen on bacterial cell physiology, biofilm-related research using 96 well plates has thus far failed to consider the role of oxygen availability and demand. Previous reports identified a maximum oxygen transfer level of 0.03 - 0.035 mol/ L / h in planktonic cultures grown a standard shaken 96-well plate (Duetz *et al.*, 2000). This may not be sufficient for growth of actively metabolizing aerobic microorganisms, and thus becomes an issue for use of the plates as "mini-bioreactors" (Samorski *et al.*, 2005). Oxygen transfer limitation is also of importance for biofilm growth, where spatial stratification of oxygen can lead to localised anoxic regions in the biofilm adjacent to the substratum (Rani *et al.*, 2007). We have previously shown that anaerobic conditions activated transcription of the *icaADBC* operon and biofilm development in *S epidermidis* by increasing the activity of the alternative sigma factor σ^B, which in turn down-regulates expression of the *icaR*

repressor (Cotter *et al.*, 2009). The results presented in this manuscript correlate well with our previous data (Cotter *et al*., 2009), as the quantity of biofilm formation was higher when the 96 well plates were incubated in an anaerobic environment. Our previous study also revealed that the *S. epidermidis rsbU* transposon mutant M15 (Mack *et al.*, 2000) was incapable of biofilm production in an anaerobic environment, but did form biofilm at high oxygen concentrations (Cotter *et al.*, 2009). Previous experiments with this *S. epidermidis* σ^B mutant were performed in 96 well plates (Knobloch *et al.*, 2001). The data presented in this study reveal that dissolved oxygen concentrations are low during bacterial growth in 96 well plate biofilm assays and therefore serve to highlight the importance of oxygen in this biofilm experimental model and indeed an important limitation of this approach.

Our data also reveal that inoculum cell concentrations influence biofilm development. A wide range of inoculum cell concentrations and culture volumes, both of which have implications for oxygen utilisation, have been used for cultivation of *S. epidermidis* biofilms in 96 well plates (Table 1). Interestingly, Sandberg *et al*. (2008) grew *S. aureus* in an aerobic environment at 200rpm, revealing an inoculum cell concentration of ~10^6 CFU ml^{-1} promoted more biofilm formation after 18 h in 96 well plates than a higher inoculum cell concentration of ~10^8 CFU ml^{-1}. Results in fig. 3, which were performed statically in a 100% oxygen environment, may differ to those conclusions due to the availability of oxygen. We conclude that 96 well plate inoculum cell concentrations influence the time at which the onset of oxygen limitation occurs, concomitantly influencing biofilm formation. This study reveals that dissolved oxygen is rapidly consumed in staphylococcal biofilm cultures grown in 96 well microtitre plates, resulting

in persistent or transient anaerobic conditions depending on the prevailing atmospheric oxygen concentrations. Given the importance of oxygen availability for bacterial physiology and biofilm formation, these data highlight a significant limitation of this technique, and it is suggested that both inoculum cell concentrations and culture volumes should be standardized in 96 well plate biofilm assays. It is also worth noting that, although not an objective of the present study, the availability of oxygen microsensors has created the potential to develop a methodology for the precise control dissolved oxygen levels in the liquid phase of microwells.

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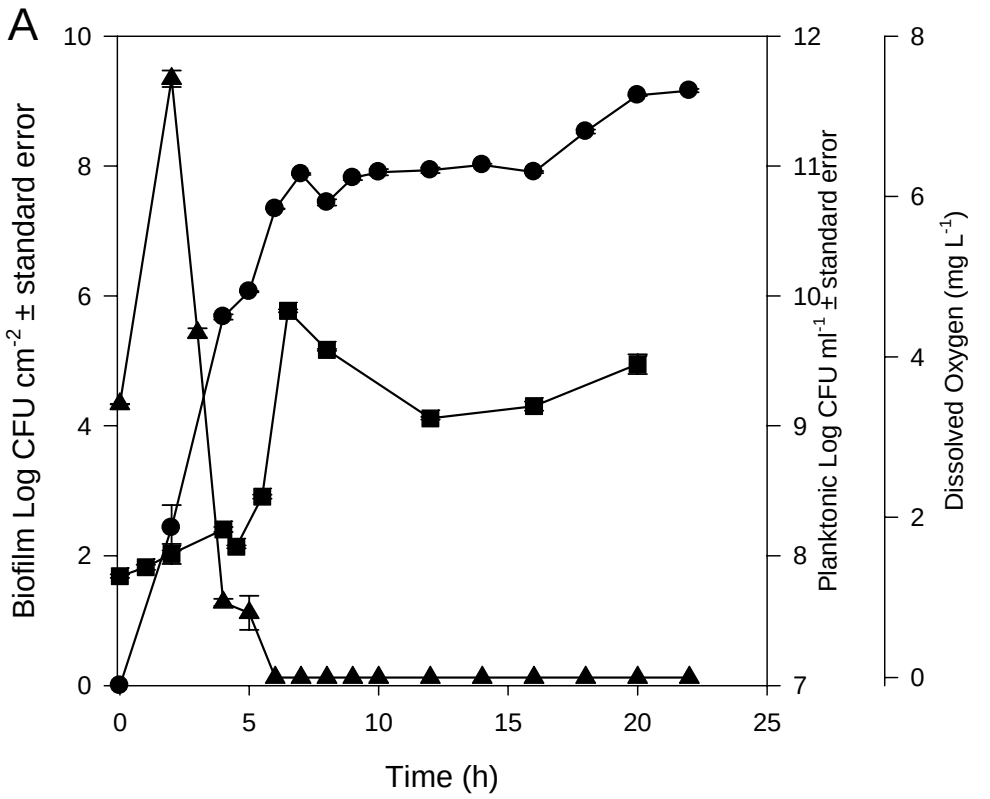
317

Figure Legends

1. Biofilm formation (●), planktonic growth (■) and dissolved oxygen (▲) in liquid cultures of *S. epidermidis* 1457, grown at 37°C for 24 h in plates incubated in air (21% oxygen) (A) or an atmosphere of 100% oxygen (B) .
2. **Influence of atmospheric oxygen concentrations on *S. epidermidis* planktonic and biofilm growth in the early hours of a 96 well biofilm assay.** *S. epidermidis* 1457 grown in 96-well plates in an atmosphere of 0% oxygen (●), 21% oxygen (○) and 100% oxygen (▼) for 6h. (A) Dissolved oxygen concentrations, (B) total biofilm formation (CFU), (C) Total bacterial cell growth (planktonic and biofilm) (CFU), and (D) ratio of planktonic to biofilm CFU counts, indicating the rate of biofilm conversion for the different oxygen concentrations. Error bars are the standard error of three wells from two independent plates.
3. **Influence of inoculum cell concentration on *S. epidermidis* biofilm production in the early hours of a 96 well plate biofilm assay.** Low (●), standard (○) and high (▼) inoculums of *S. epidermidis* 1457 grown in 96-well plates, incubated in an atmosphere of 100% oxygen. (A) Dissolved oxygen, (B) total biofilm formation (CFU), (C) Total growth of biofilm + planktonic CFU, and (D) ratio of planktonic to biofilm CFU counts.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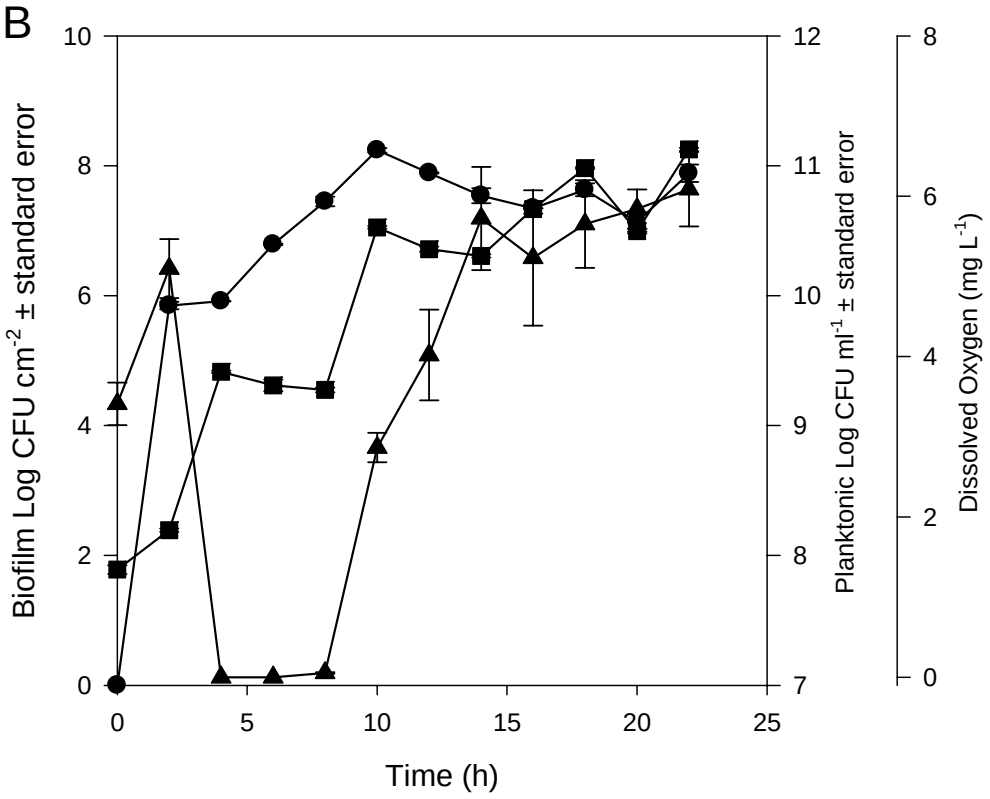
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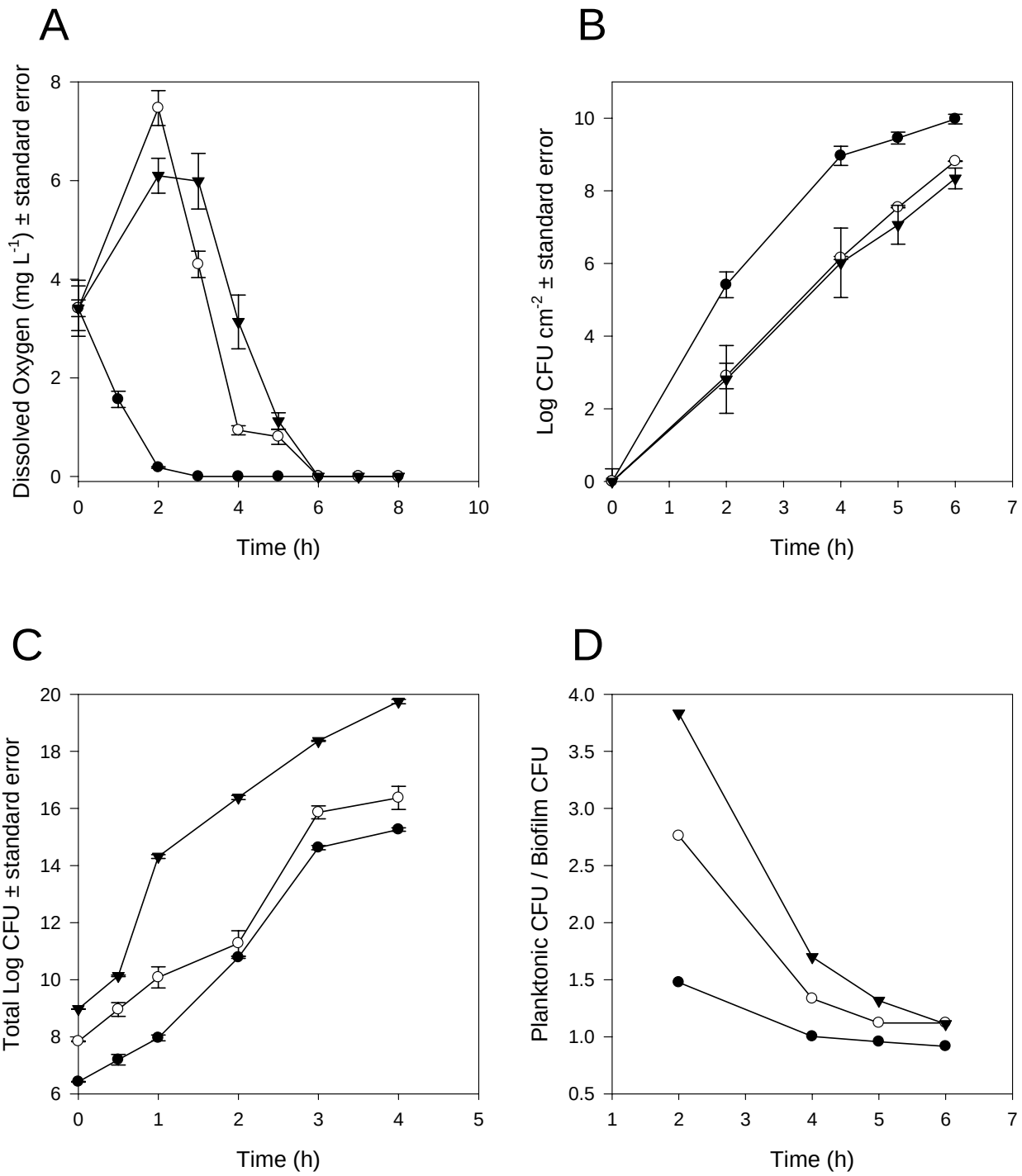
339 Fig. 1



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342 Fig. 2

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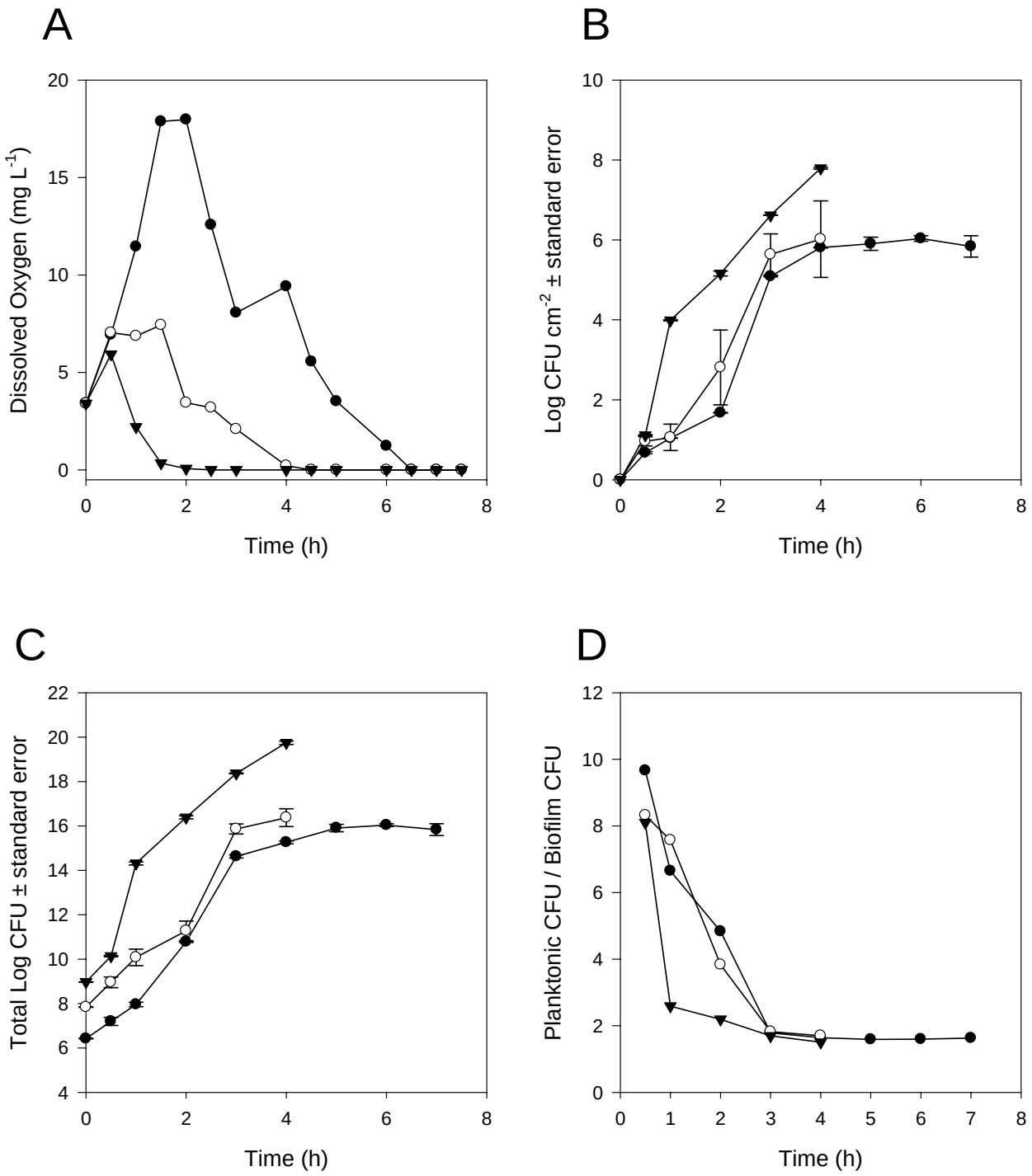

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346 Fig. 3

Table 1. A selection of inoculum cell concentrations and culture volumes of *Staphylococcus epidermidis* strains used in 96 well plate biofilm assays.

Strain	Inoculum	Culture Volume	Reference
ATCC 35984	4×10^7- 1×10^9 CFU ml^{-1}	100 µl	Dunne *et al.*, 1991
CSF 41498	Undiluted overnight culture	100 µl	Conlon *et al.*, 2002
ATCC 35984	10% of overnight culture	200 µl	Peeters *et al.*, 2008
ATCC 35983, 35984, 35981,35982	dilution of overnight culture	200 µl	Christensen *et al.*, 1985
ATCC 55113, SE1175	1: 50 dilution of overnight culture	200 µl	Wu *et al.*, 2003
1457, NJ9709	10^3- 10^5 CFU ml^{-1}	200 µl	Izano *et al.*, 2008
1457, ATCC 35984	1:200 dilution of overnight culture	200 µl	Heilmann *et al.*, 1996

Table 1