



Research Repository UCD

Title	Rapid depletion of dissolved oxygen in 96 well microtitre plate Staphylococcus epidermidis biofilm assays promotes biofilm development and is influenced by inoculum cell concentration
Authors(s)	Cotter, John J., O'Gara, James P., Casey, Eoin
Publication date	2009-08-01
Publication information	Cotter, John J., James P. O'Gara, and Eoin Casey. "Rapid Depletion of Dissolved Oxygen in 96 Well Microtitre Plate Staphylococcus Epidermidis Biofilm Assays Promotes Biofilm Development and Is Influenced by Inoculum Cell Concentration" 103, no. 5 (August 1, 2009).
Publisher	Wiley
Item record/more information	http://hdl.handle.net/10197/2729
Publisher's statement	This is the authors' version of the following article: "Rapid depletion of dissolved oxygen in 96-well microtiter plate Staphylococcus epidermidis biofilm assays promotes biofilm development and is influenced by inoculum cell concentration" published in Biotechnology and Bioengineering 103:5 pp 1042–1047. It is available in its final form at http://dx.doi.org/10.1002/bit.22335
Publisher's version (DOI)	10.1002/bit.22335

Downloaded 2024-04-18 16:13:04

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)



© Some rights reserved. For more information

**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

John J. Cotter¹, James P. O’Gara² and Eoin Casey^{1*}.

¹UCD School of Chemical and Bioprocess Engineering, Centre for Synthesis and
Chemical Biology, and ²UCD School of Biomolecular and Biomedical Science and UCD
Conway Institute of Biomolecular and Biomedical Research, University College Dublin,
Belfield, Dublin 4, Ireland.

*Corresponding Author: Eoin Casey.

Address: UCD School of Chemical and Bioprocess Engineering, Engineering and
Materials Science Centre, University College Dublin, Belfield, Dublin 4, Ireland.

Email: eoin.casey@ucd.ie

Telephone: +353 1 7161877

Fax: +353 1 7161177

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

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

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

55 Polystyrene 96-well plates have become one of the most popular methods for micro scale
56 biofilm investigations (Christensen *et al.*, 1985) and are commonly used in biofilm
57 genetic studies (Davey and O'Toole, 2000) to analyse differences in the quantity of
58 biofilm formation in engineered mutants. A downside of this technique is the
59 considerable experiment to experiment variation in biofilm production by individual
60 bacterial strains. Nevertheless the technique is useful in determining whether bacterial
61 strains are capable of forming biofilm (Peeters *et al.*, 2008). Specific modifications have
62 been made to the basic 96 well plate method (Christensen *et al.*, 1985) by different
63 groups, particularly in terms of the inoculum cell concentration and culture volumes.
64 Table 1 indicates the range of inoculum cell concentrations and culture volumes used in
65 *Staphylococcus epidermidis* 96 well plate biofilm assays. Biofilm experiments using 96
66 well plates are typically incubated at the optimal growth temperature without shaking and
67 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 $\log 7.84 \pm 0.01$ colony forming units (CFU) ml^{-1} . Experiments were also undertaken using lower and higher inoculum cell concentrations. The “low” inoculum consisted of $\log 6.43 \pm 0.02$ 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 $\sim 7 \text{ mg L}^{-1}$ 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

planktonic cells at different oxygen concentrations are shown in Fig. 2. The total biofilm formation of *S. epidermidis* 1457 in each well is shown as “Total biofilm CFU” in this figure. The initial dissolved oxygen concentration is that of autoclaved BHI media and is the same for all profiles. In an atmosphere of 0% or 21% oxygen, when dissolved oxygen was consumed it remained below detection for the remainder of the experiment (up to 24 h), though only the initial hours are shown in Fig. 2. A profile of dissolved oxygen concentrations in 96 well plates incubated in an atmosphere of 100% oxygen conditions is shown in Fig. 1B. The ratios of planktonic to biofilm cell counts in 96 well plates incubated in an atmosphere of 0%, 21% or 100% oxygen are shown in Fig. 2D. Predictably, at higher atmospheric concentrations of oxygen, the dissolved oxygen concentrations in 96 well plates take longer to be depleted. Biofilm cell numbers were statistically significantly higher under anaerobic conditions compared to 21% and 100% oxygen conditions ($p < 0.05$). Indeed, the ratio of planktonic to biofilm cell numbers revealed that overall in the 96 well plate cultures a higher fraction of the biomass exists as biofilm under anaerobic compared to aerobic conditions.

Inoculum cell concentrations influence oxygen consumption and biofilm formation.

Three different initial cell concentrations (low, standard and high inocula) of *S. epidermidis* 1457 were used to inoculate 96 well plate cultures in an atmosphere of 100% oxygen. Fig. 3 shows dissolved oxygen concentrations, biofilm cell numbers, total bacterial cell numbers and the ratio of planktonic to biofilm cell numbers under these conditions. The ratio of planktonic to biofilm cell numbers reaches 1.4 ± 0.15 after 4 h irrespective of the inoculum cell concentration. The oxygen demand in the first few 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 in 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 $\sim 10^6$ CFU ml⁻¹ promoted more biofilm formation after 18 h in 96 well plates than a higher inoculum cell concentration of $\sim 10^8$ 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.

Acknowledgements

This research was funded by Science Foundation Ireland (SFI) grant 04/BRG/E0072. We thank Eoin Syron and Sharon Davin for technical support. Strain *S. epidermidis* 1457 was a kind gift from Dietrich Mack, University of Swansea, Wales, U.K.

References

1. Bjergbæk LA, Haagensen JAJ, Reisner A, Molin S, and Roslev P. 2006. Effect of oxygen and growth medium on in vitro biofilm formation by *Escherichia coli*. *Biofilms* 3: 1-10.
2. Burmolle M, Webb JS, Rao D, Hansen LH, Sorensen SJ and Kjelleberg S. 2006. Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. *Appl. Environ. Microbiol.* 72: 3916-3923.
3. Characklis WG and Cooksey KE. 1983. Biofilms and Microbial Fouling. *Adv. Appl. Microbiol.* 29: 93-138.
4. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM and Beachey EH. 1985. Adherence of Coagulase-Negative Staphylococci to Plastic Rissue Culture Plates: a Quantative Model for the Adherence of Staphylococci to Medical Devices. *J. Clin. Microbiol.* 22: 996-1006.
5. Conlon KM, Humphreys H and O'Gara JP. 2002. *icaR* Encodes a Transcriptional Repressor Involved in Environmental Regulation of *ica* Operon Expression and Biofilm Formation in *Staphylococcus epidermidis*. *J. Bacteriol.* 184: 4400-4408.
6. Cotter JJ, O'Gara JP, Mack D and Casey E. 2009. Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor σ^B in *Staphylococcus epidermidis*. *Appl. Environ. Microbiol.* 75: 261-264.

7. Cramton SE, Ulrich M, Gotz F and Doring G. 2001. Anaerobic Conditions Induce Expression of Polysaccharide Intercellular Adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*. Infect. Immun. 69:4079-4085.
8. Davey ME and O'Toole GA. 2000. Microbial Biofilms: from Ecology to Molecular Genetics. Microbiol. Mol. Biol. Rev. 64: 847-867.
9. Duetz WA, Ruedi L, Hermann R, O'Connor K, Büchs J and Witholt B. 2000. Methods for Intense Aeration, Growth, Storage, and Replication of Bacterial Strains in Microtiter Plates. Appl. Environ. Microbiol. 66: 2641-2646.
10. Dunne WM, Jr. and Burd EM. 1991. In vitro measurement of the adherence of *Staphylococcus epidermidis* to plastic by using cellular urease as a marker. Appl. Environ. Microbiol. 57: 863-866.
11. Goeres DM, Loetterle LR, Hamilton MA, Murga R, Kirby DW and Donlan RM. 2005. Statistical assessment of a laboratory method for growing biofilms. Microbiol. 151: 757-762.
12. Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D and Gotz F. 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol. Microbiol. 20:1083-1091.
13. Izano EA, Amarante MA, Kher WB and Kaplan JB. 2008. Differential Roles of Poly-N-Acetylglucosamine Surface Polysaccharide and Extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms. Appl. Environ. Microbiol. 74: 470-476.

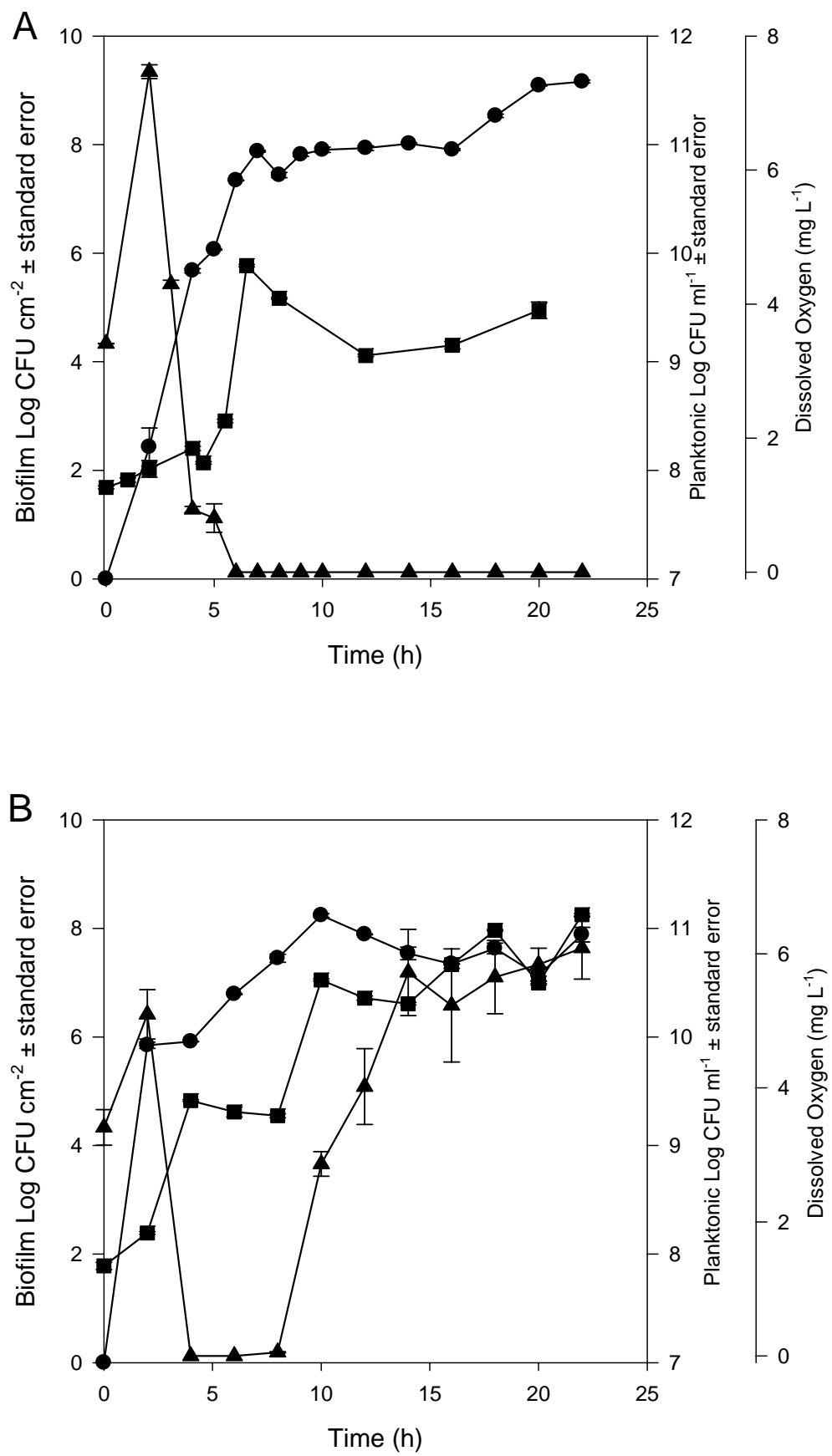
14. Knobloch JKM, Bartscht K, Sabottke A, Rohde H, Feucht H. H and Mack D. 2001. Biofilm Formation by *Staphylococcus epidermidis* Depends on Functional RsbU, an Activator of the *sigB* Operon: Differential Activation Mechanisms Due to Ethanol and Salt Stress. J. Bacteriol. 183: 2624-2633.
15. Mack D, Siemssen N, and Laufs R 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. Infect. Immun. 60: 2048-2057.
16. Mack D, Rohde H, Dobinsky S, Riedewald J, Nedelmann M, Knobloch JKM, Elsner HA and Feucht HH. 2000. Identification of Three Essential Regulatory Gene Loci Governing Expression of *Staphylococcus epidermidis* Polysaccharide Intercellular Adhesin and Biofilm Formation. Infect. Immun. 68: 3799-3807.
17. Micheletti M, Barrett T, Doig SD, Baganz F, Levy MS, Woodley JM and Lye GJ. 2006. Fluid mixing in shaken bioreactors: Implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures. Chem. Eng. Sci. 61: 2939-2949.
18. O'Gara JP. 2007. *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. FEMS Microbiol. Lett. 270: 179-188.

19. Peeters E, Nelis HJ and Coenye T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J. Microbiol. Methods 72: 157-165.
20. Pitts B, Hamilton MA, Zilver N and Stewart PS. 2003. A microtiter-plate screening method for biofilm disinfection and removal. J. Microbiol. Methods 54: 269-276.
21. Rani SA, Pitts B, Beyenal H, Veluchamy RA, Lewandowski Z, Davison WM, Buckingham-Meyer K, and Stewart PS. 2007. Spatial Patterns of DNA Replication, Protein Synthesis, and Oxygen Concentration within Bacterial Biofilms Reveal Diverse Physiological States. J. Bacteriol. 189: 4223-4233.
22. Samorski M, Muller-Newen G, and Büchs J. 2005. Quasi-continuous combined scattered light and fluorescence measurements: A novel measurement technique for shaken microtiter plates. Biotech. Bioeng. 92: 61-68.
23. Sandberg M, Määttänen A, Peltonen J, Vuorela PM, and Fallarero A. 2008. Automating a 96-well microtitre plate model for *Staphylococcus aureus* biofilms: an approach to screening of natural antimicrobial compounds. Int. J. Antimicrob. Agents 32: 233-240.
24. Shin CS, Hong MS and Lee J. 1996. Oxygen transfer correlation in high cell culture of recombinant *Escherchia coli*. Biotechnol. Tech. 10: 679-682.
25. Stewart PS and Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358: 135-138.

26. Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, and Costerton JW. 2001. Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. *Appl. Environ. Microbiol.* 67: 5608-5613.
27. Wu JA, Kusuma C, Mond JJ, and Kokai-Kun JF. 2003. Lysostaphin Disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* Biofilms on Artificial Surfaces. *Antimicrob. Agents Chemother.* 47: 3407-3414.
28. Xu KD, McFeters GA, and Stewart PS. 2000. Biofilm resistance to antimicrobial agents. *Microbiol.* 146: 547-549.
29. Zilver N, Hamilton M, Pitts B, Goeres D, Walker D, Sturman P, and Heersink J. 1999. Measuring antimicrobial effects on biofilm bacteria: From laboratory to field, p. 608-628. *Methods in Enzymology*. Academic Press.
30. Zhang H, Lamping SR, Pickering SCR, Lye GJ and Shamlou PA 2008. Engineering characterisation of a single well from 24-well and 96-well microtitre plates. *Biochem. Eng. J.* 40:138-149.

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.



338

339 Fig. 1

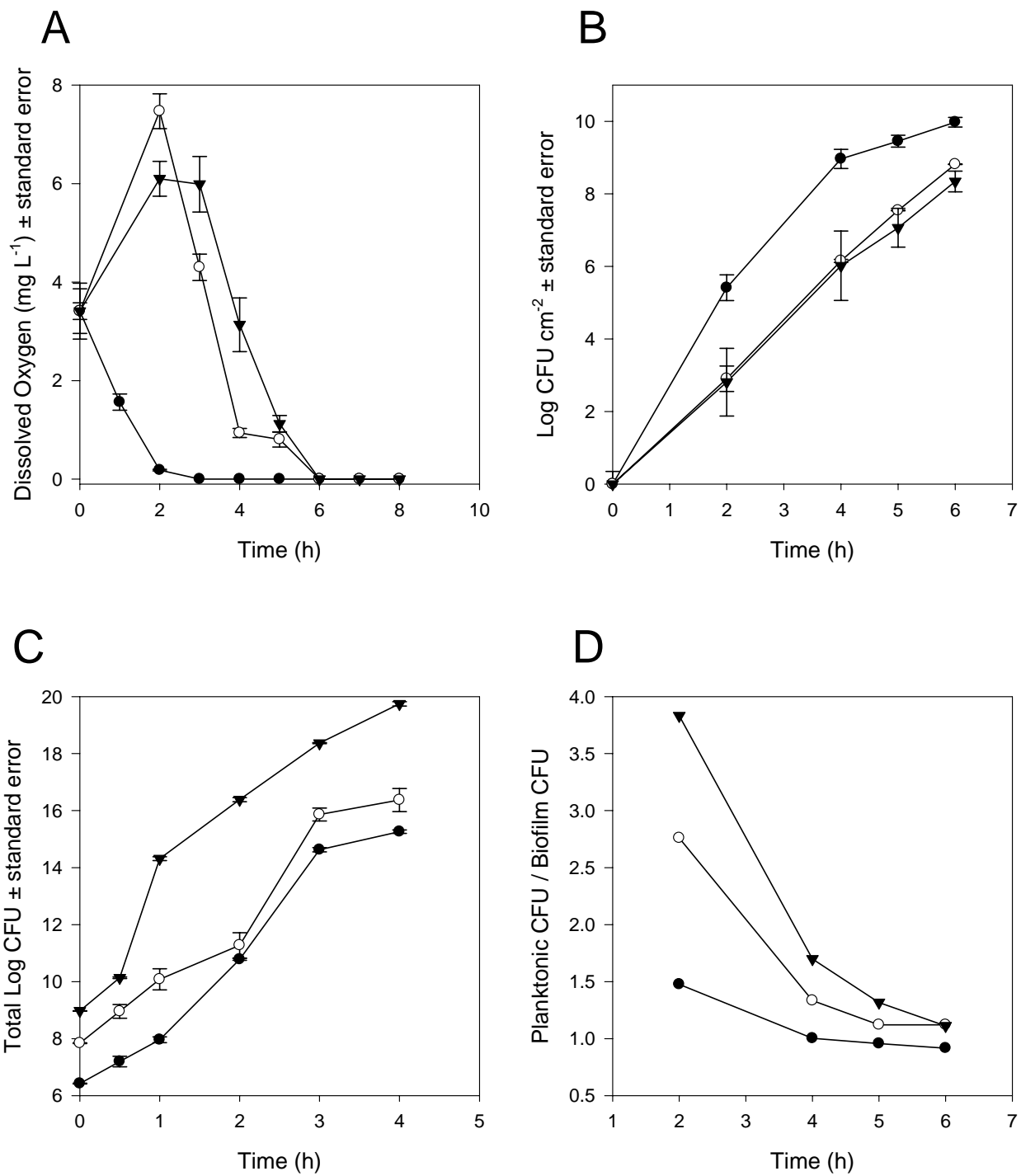


Fig. 2

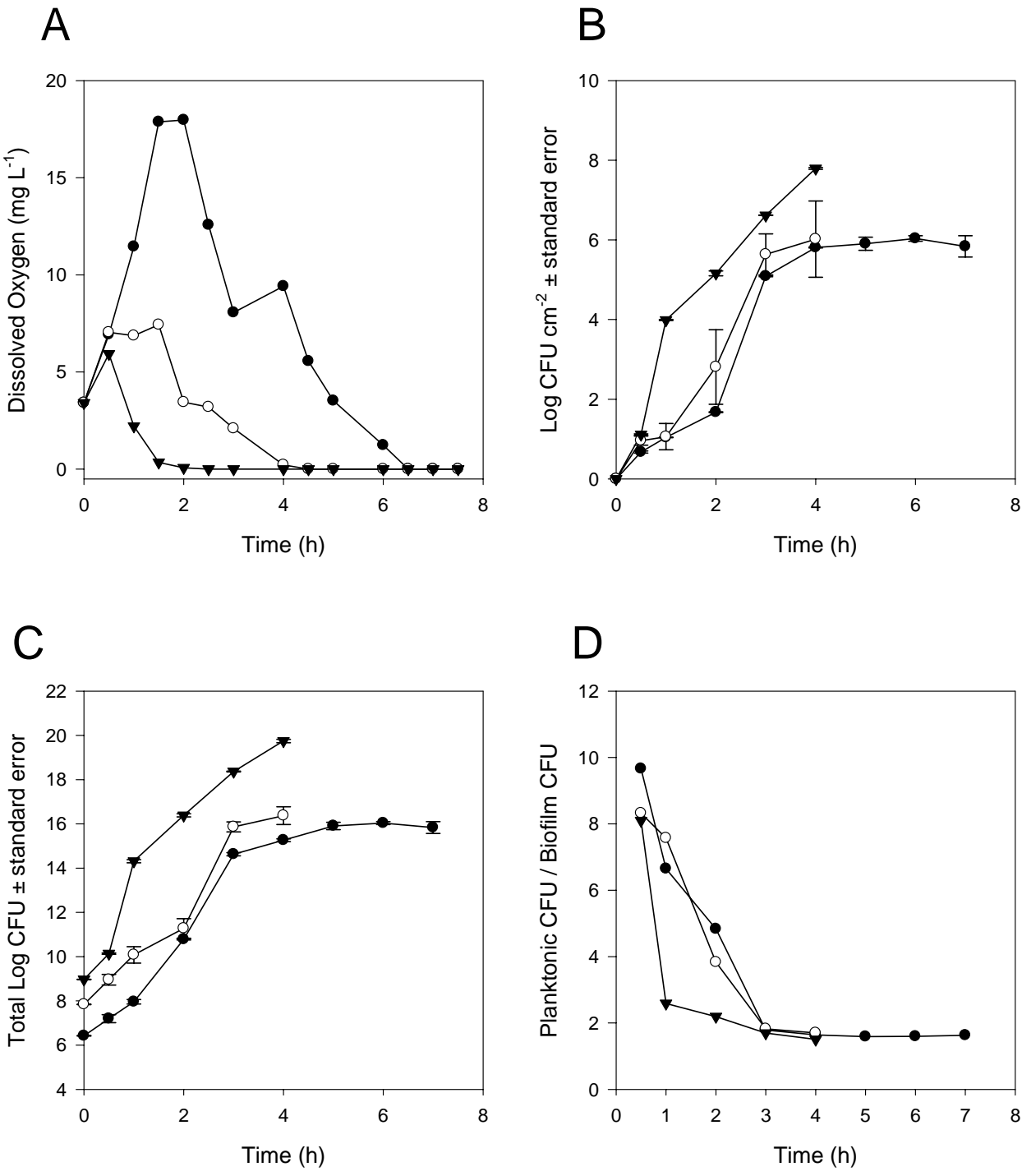


Table 1. A selection of inoculum cell concentrations and culture volumes of *Staphylococcus epidermidis* strains used in 96 well plate biofilm assays.

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

Table 1