Characterisation of a modified Rotating Disk Reactor for the cultivation of *Staphylococcus epidermidis* biofilm

Running title: A modified RDR for *S. epidermidis* biofilm cultivation

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Abstract

Aims: The purpose of this study was to develop a system that would allow biofilms to be cultivated under strictly defined conditions in terms of dissolved oxygen, fluid shear and to assess if the method was suitable for detection of respiratory activity stratification in biofilm samples.

Methods: The system is a modified version a commercially available laboratory biofilm reactor and incorporates a number of features such as provision of defined levels of dissolved oxygen, constant average shear, enhanced gas-liquid mass transfer, aseptic operation and the ability to remove biofilm for ex-situ analysis during or after continuous cultivation.

Conclusions: The system was shown to be effective for the characterisation of the effects of dissolved oxygen on a pure culture of *Staphylococcus epidermidis*. The versatility of the system offers the potential for cultivating pure culture biofilm in defined, controlled conditions and facilitates a range of analyses that can be performed ex situ.

Significant and impact of study: The ability to provide strict regulation of environmental conditions and enhanced transfer of oxygen to the biofilm during cultivation are important, firstly because oxygen is known to regulate biofilm development in several microorganisms and secondly because many conventional biofilm cultivation systems may not provide adequate oxygen supply to the biofilm.

Keywords: biofilm, reactor, cultivation, oxygen, sensor, antibiotics, shear, *Staphylococcus epidermidis*.

Introduction

The interaction between a biofilm and the environment in which it is grown is not completely understood. The biofilm mode of growth is believed to be a form of survival, both in an environmental or controlled laboratory setting (Donlan and Costerton, 2002). From the surface of skin to a potentially anaerobic environment in the depths of an artificial implant, the bacteria that constitute a biofilm encounters a wide range of oxygen levels (Rowlinson *et al*., 2006). The majority of investigations into the influence of controlled oxygen levels on growth and antibiotic susceptibility of a biofilm have focused on *Pseudomonas aeruginosa* (Borriello *et al*., 2004; Fonseca and Sousa, 2007). In *Staphylococcus epidermidis* biofilm, oxygen gradients correspond to DNA synthesis activity (Rani *et al*., 2007), and gradients in nutrient concentration have been identified as a factor in the increased susceptibility to antibiotics (Zheng and Stewart, 2002). Difficulty in eradication of biofilm infection using common therapeutic means is well documented (Stewart and Costerton, 2001), but due to a lack of tight environmental regulation in many *in vitro* systems, the influence of oxygen gradients on biofilm development and antibiotic susceptibility are unclear.

One obstacle in controlling the formation and development of a biofilm is the lack of a fundamental understanding of mechanisms associated with the formation and effects of physiological heterogeneity. Local micro-environmental conditions in the biofilm play an important role in the behaviour of the biofilm by determining not only reaction rates but also through induction of biological responses to sub-optimal conditions. Spatial physico-chemical gradients determine local conditions in biofilms and, therefore, physiological heterogeneity is inevitable. It is generally accepted that the existence of physiological

68 heterogeneity in biofilms arises mainly as a consequence of nutrient gradients formed by
69 the reaction-diffusion mechanism (Stewart, 2002; Werner *et al*., 2004). Due to its low
70 solubility, oxygen is frequently the limiting nutrient in biofilms. Studies have shown that
71 oxygen limitation and consequent low metabolic activity in the interior of the biofilm are
72 correlated with antibiotic tolerance in *Pseudomonas aeruginosa* (Walters *et al*., 2003;
73 Rani *et al*., 2007).
74 This work initially sought to characterise a biofilm reactor, known as the rotating disk
75 reactor (RDR), commercially available from Biosurface Technologies Corp. (Bozeman,
76 MO, USA), as a means to investigate the effect of oxygen on the growth and antibiotic
77 susceptibility of *S. epidermidis* biofilm. A number of other lab based systems have been
78 used to cultivate *S. epidermidis* biofilm, such as the CDC reactor (Del Pozo *et al*., 2009)
79 and glass flow cells (Rani et al., 2005). The commercial RDR has been used for
80 monoculture biofilms, as shown in the case of *P. aeruginosa* (Hentzer *et al*., 2001), but it
81 can also be utilized for mixed culture samples (Zelver *et al*., 2001). Subsequently, the
82 RDR was developed as an American Society for Testing Materials (ASTM) Standard
83 Method (Designation E2196), for quantification of *P. aeruginosa* biofilm grown in shear
84 and continuous conditions. Table 1 summarizes details of published studies where the
85 commercial RDR was used to produce biofilm. A second phase of this work was to
86 develop and characterise a modified RDR system which was designed to provide
87 improved gas-liquid mass transfer and mixing. The primary objective of this part of the
88 study was to investigate if the modified RDR would allow biofilms to be cultivated under
89 strictly defined conditions in terms of oxygen levels, fluid shear and aseptic operation,

and to assess if the method was suitable for detection of respiratory activity stratification in the biofilm.

Materials and Methods

Bacterial strains 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 50ml brain heart infusion (BHI, Oxoid, U.K.) broth overnight in an incubated orbital shaker at 37°C and 150 rpm without aeration. This strain was a kind gift from Dietrich Mack to the O'Gara laboratory.

The Rotating Disk biofilm Reactor (RDR)

Both the commercial rotating-disk reactor (designated as cRDR) and a modified rotating-disk reactor (mRDR) were used in this study. The cRDR; (Biosurface Technologies Corp, MT, USA), consists of a 1-litre glass vessel with a waste outlet located one-quarter of the height (~40 mm) from the bottom of the reactor. This provides a working volume of approximately 180 ml when the disk is in place and rotating, though this reduces with increasing speeds of rotation due to vortex formation. The lid has an inoculation port and three ports for liquid medium or gas input. The RDR can be operated in batch or continuous flow and the velocity field under which the biofilm is formed is adjustable by means of the magnetically driven rotating disk (diameter 70 mm), which sits at the bottom of the reactor vessel. All rotational speeds reported were verified using a tachometer prior to initiation of operation. Rotation of the disk provides continuous

113 mixing in the system. The disk holds six coupons in place (diameter 12.7 mm), which
114 are inserted prior to autoclaving. In these experiments polycarbonate coupons were used.
115 The disk is removed by a sterile hook when the biofilm is to be analysed. The entire
116 system, without the peristaltic pump, was autoclaved prior to use at 121°C for 15 min.
117 The reactor and feed were placed in an incubator at 37°C.
118
119 **Modified RDR (mRDR)**
120 The commercial RDR was modified to facilitate input of gas directly into the liquid
121 media. A stainless steel sparger (inner diameter 4mm, outer diameter 7mm) extended the
122 gas input port into the liquid, allowing gas to be sparged directly into the liquid. The
123 sparger was widened at the bottom (to 15 mm, for the bottom 19 mm of the pipe) to
124 simultaneously act as a baffle in the system, reducing the vortex formed when the disk
125 rotates. The lengths of the sparger were specified so that if gas was input through it,
126 bubbles would not interfere with the rotation of, or scour any biofilm adhering to, the
127 surface of the disk. The mRDR and cRDR are shown schematically in fig. 1.
128
129 **Cultivation procedure applied to both cRDR and mRDR**
130 The reactors were filled with BHI (Oxoid, Basingstoke, Hampshire, UK) and sterilized by
131 autoclaving and subsequently placed in an incubator at 37°C ± 1°C. 150 ml of liquid
132 BHI was used for the cRDR reactors, with 180ml used for mRDRs. The difference in
133 this starting volume relates to the influence the vortex has on the liquid volume in the
134 reactor, and is explained in the discussion section. A 1% (v/v) solution of an overnight
135 culture (adjusted to A_{660}= 1.0) was used to inoculate each reactor. This concentration of

cells equated to ~10^5 CFU ml^{-1}. For continuous operation, quarter-strength BHI was drip fed from the lid into the reactor. Using a small modification to the tubing on the waste line, samples of planktonic cells were removed for quantification over time. Samples were taken throughout a 48 h run, with offline optical density measurements at 660nm ($O.D._{660nm}$) and colony forming units assessed at each time point.

Gas of known oxygen concentrations were sparged into mRDR. Nitrogen and air (Air Products, Dublin) were blended in a gas mixing station, with oxygen concentration verification performed offline using a gas analyser prior to the commencement of every run. Gas was sparged at 0.5 L min^{-1} throughout all runs. Concentrations of 0%, 7%, 14% and 21% oxygen were examined for their influence on biofilm formation. Most comparisons were performed between the 0% and 21% oxygen sparged biofilms.

Monitoring of Dissolved Oxygen

As a means of assessing oxygen utilization in the modified reactor by *S. epidermidis* 1457, fluorometric oxygen sensor spots (PreSens GmbH, Regensburg, Germany) were used to record the dissolved oxygen levels within the RDR during experiments where the sparged gas contained 0 and 21% oxygen. The sensor spots (Puskeiler *et al.,* 2005) were adhered to the inner wall of the RDR between the air-liquid interface and the disk using a minimal amount of silicone rubber compound 692–542 (RS Components, Corby, U.K.), and transmitted readings to a detector outside of the reactor. This method to measure oxygen via dynamic fluorescence quenching allows in situ measurement of dissolved oxygen concentrations in aqueous solutions. The spots, which have an area of 28 mm^2, are designed to operate through glass or any transparent substance, and were sterilised in

159 place as the reactor was autoclaved. The accuracy of the spots is ± 0.4% O_2 at 20.9% O_2
160 and ± 0.05% O_2 at 0.2% O_2 (Liebsch *et al*., 2000). The range of detection is 0- 45 mg L^{-1}.
161

162 **Volumetric mass transfer calculations for the cRDR and mRDR**

163 To calculate a value for the volumetric mass transfer in the cRDR and mRDR, nitrogen
164 was pumped into each reactor under normal operating conditions (37ºC) using sterilised
165 H_2O as media and without bacterial inoculation. If a near saturated liquid mass is
166 contacted with an oxygen-free gas e.g., pure nitrogen, then desorption will occur with
167 oxygen transferring from the oxygen rich liquid to the sparging gas. An oxygen balance
168 on the liquid for a short time *dt* yields;

169 $$-d(VC) = k_\ell aVCdt$$

170 where *a* refers to the interfacial area per unit volume of liquid. Integration of the above
171 equation between time t = 0 and time t = t, when the oxygen concentrations are C_o (often
172 zero) and C(t) respectively, yields,
173

174 $$\ln\frac{C_o}{C(t)} = k_\ell at$$

175 a semi-log plot will yield a value for $k_l a$.
176

177 **Quantification of biofilm**

178 The variation of biofilm CFUs adhering to coupons in the same reactor, and coupons in
179 different reactors under identical conditions, were assessed. Six coupon spaces within
180 one rotating disk ensures that the repeatability of biofilm formation within one reactor
181 can be easily analysed. To compare biofilm formation under the different reactors, each

type of RDR was run in one incubator with the same inoculum, media and disk rotational speed.

To quantify biofilm adherence, coupons from two cRDRs and two mRDRs, thus 12 coupons from each type of reactor, were removed from the reactor, washed and placed in 9 ml sterile Ringers (Hall-Stoodley and Lappin-Scott, 1998). For the sonication method, the coupons in Ringers were vortexed for 5 min, sonicated for 2 min and then vortexed again for 2 min. The sonication was designed to lift the biofilm from the surface of the coupon, and the vortexing was optimised to provide maximum disruption of the aggregates to create a uniform solution which could be serially diluted and plated on nutrient agar to assess the colony forming units. Plates were incubated at 37°C for 24 h. Statistical comparisons were performed using the Student's t-test, and CFU calculations according to Zelver *et al*. (1999).

Influence of Disk rotational speed on biofilm formation

Modifying the operation of a cRDR to produce biofilm under different shear conditions relies on the interaction between the disk and the magnetic stirrer. Three rotational speed settings were examined for *S. epidermidis* biofilm formation: low (100 rpm) medium (150 rpm) and high (200 rpm) settings. The Reynolds numbers were 8,167, 12,250, 16,333 for the rotational speeds 100, 150 and 200 rpm respectively. All rotational speeds were verified prior to inoculation using a tachometer (Tm-3011 tachometer; Veeder Root, Richmond, U.K.). Experiments where the rotational speed was above 200 rpm are not reported because the unstable nature of the disk rotation over an extended period of time, such as 24 h, frequently led to the disk stopping before the conclusion of operation. The

205 interaction between the magnetic stirrer and the disk is a limitation of this system, which
206 requires careful consideration prior to selecting a rotational speed.
207
208 **Antibiotic susceptibility of *S. epidermidis* biofilm grown under different oxygen**
209 **concentrations**
210 In these experiments rifampicin treatment of biofilm samples were analysed for log
211 reduction in CFU counts after 24 h of treatment. Samples were removed from the RDR
212 after 48 h growth in different sparged oxygen conditions and treated with 2 μg ml^{-1}, 1 μg
213 ml^{-1} and 0.5 μg ml^{-1} for 24 h at 37°C. These experiments were performed to investigate
214 if the sparged oxygen concentration in which the biofilm was cultivated had an influence
215 on the antibiotic susceptibility of the cells.
216
217 **Application of fluorescent stains**
218 For mRDR coupons, biofilms were washed three times in Ringers to remove any
219 planktonic cells. Samples were stained with 5-cyano-2, 3-ditolyl tetrazolium chloride
220 (CTC), a tetrazolium salt which is reduced to a fluorescent derivative indicating redox
221 activity in a biofilm. It has previously been used as an indicator of respiratory activity in
222 bacteria (Rabinovitch and Stewart, 2006), and a final concentration of 0.04% in deionised
223 autoclaved water was applied directly to the coupon, prior to incubation at 37°C for 1 h
224 in the dark (Yu and McFetters, 1994). Samples were dipped in sterile ringers after this
225 time. For this method, as with all the analysis methods used in these experiments, the
226 biofilms adhered to the coupons were sacrificed and were not used for other analyses.
227 These experiments were only performed on mRDR biofilms as the oxygen levels in

228 which the biofilm was cultured was known, rather than in the cRDR where no attempt is
229 made to control the environment.
230
231 **Cryoembedding biofilms**
232 To prevent damage occurring to a sample exposed to freezing conditions, biofilms were
233 embedded in a protective medium (O.C.T. Tissue-tek embedding compound, Sakura, The
234 Netherlands) for 10 min after staining. This allows the O.C.T. time for complete
235 penetration of the biofilm and maximum protection. For coupon samples the embedded
236 biofilm is placed into a chamber of containing n-hexane surrounded by liquid carbon
237 dioxide for 10 min according to the procedure outlined by Fox *et al*. (1995). Biofilms
238 were stored at -80ºC prior to sectioning. Biofilms were stored in triplicate for each of the
239 subsequent analyses.
240 **Cryosectioning**
241 Cryosectioning was performed at –23°C (Yu *et al*., 1994) in a Microm HM500 (Microm,
242 Walldorf, Germany). For biofilms attached to coupons, the substratum was removed
243 prior to the biofilm being mounted on the slicing stage. The samples were positioned to
244 enable a cross section of the biofilm to be taken. Slices of 5μm were cut and stored on
245 Poly-L-Lysine coated slides prior to counterstaining with 100 μg ml^{-1} of 4', 6-diamidino-
246 2-phenylindole (DAPI) in the dark for 3 min. Poly-L-Lysine is used to create an
247 attraction between the slides and the sample, and in this method DAPI is a counterstain to
248 the previously used CTC, which indicates areas of respiratory activity only. Slides were
249 viewed using an Olympus BX51 fluorescent microscope (Olympus, Japan) at 40x
250 magnification. Fluorescent images were overlaid using Adobe Photoshop (Adobe

Systems, CA, U.S.A.), and thickness measurements were calculated using Able Image Analyser (Mu Labs, Slovenia).

Results

Influence of Disk Rotational speed on biofilm formation

Three rotational speed settings were examined for *S. epidermidis* biofilm formation in the commercial RDR, with an inversely proportional relationship identified between rotational speed and biofilm quantity. The greatest biofilm accumulation occurred at the lowest speed examined, 100 rpm, where 9.16 ± 0.2 log CFU cm^{-2} of biofilm adhered to the coupons. Experiments at 150 rpm gave 8.05 ± 0.31 log CFU cm^{-2} biofilm and 200 rpm gave 7.66 ± 0.35 log CFU cm^{-2}. Fonseca and Sousa (2007) noted that shear had a negative effect on biofilm formation in *P. aeruginosa*, which correlates with our results. For the purposes of maintaining consistency in subsequent investigations, 200 rpm was chosen as the rotational speed of the disk. Although the exact value of shear encountered in *in vivo* biofilms is unknown it is known that biofilms in, for example, artificial implants do not necessary encounter static conditions (Costerton *et al*., 2007).

Biofilm formation in anaerobic atmospheric conditions

To examine the influence anaerobic conditions have on *S. epidermidis* biofilm formation, filtered nitrogen gas (Air Products, Dublin) was pumped into the headspace of the cRDR at 0.5 L ml^{-1} for the duration of a 24 h batch run. Biofilm formed in greater amounts with nitrogen pumped into the headspace, with 8.31 ± 0.19 log CFU cm^{-2} biofilm forming on coupons in anaerobic conditions compared to 7.65 ± 0.14 log CFU cm^{-2} biofilm in non-

aerated conditions. This is a statistically significant difference in biofilm formation on the coupons in terms of culturable cells ($p < 0.05$). Biofilm formation occurred at the interface between the disk, liquid and headspace (not shown), and this accumulation was a factor in the construction of the sparger with a widened base to alter the hydrodynamic conditions and reduce the size of the vortex formed by the rotation of the disk.

Biofilm formation under various sparged oxygen conditions in the mRDR

To quantify planktonic cell growth, samples were taken throughout a 48 h run and analysed for O.D. and viable cells. Fig. 2A shows planktonic growth for a nitrogen sparged system, and Fig. 2B for a 21% oxygen sparged system. The CFU counts for both are similar, particularly when the oxygen in consumed in the 21% sparged oxygen reactors (fig. 2B), but the optical density of the planktonic liquid is greater for the 21% oxygen reactors. In a dynamic system such as the modified RDR, attachment and reattachment of biofilm is expected to be ongoing throughout the duration of a run, but with biofilm formation greater under anaerobic conditions, this may explain why the O.D. in nitrogen sparged conditions is so much lower than in 21% oxygen conditions.

Oxygen mass transfer rate measurements

Using the sensor spots to assess dissolved oxygen in the reactor under abiotic conditions, calculations of the volumetric transfer of oxygen under different rotational speeds were performed for the cRDR and mRDR (Table 2). The modifications to input gas directly into the media in the mRDR have a considerable effect on the transfer of oxygen into the

296 media compared to the cRDR, with a 10 fold difference in volumetric mass transfer at
297 200 rpm.
298
299 **Dissolved oxygen concentration in liquid**
300 Sensor spots on the inner wall of the modified reactor were positioned to give direct
301 readings of dissolved oxygen from the liquid medium. Fig. 3 displays the profiles for
302 0%, 21% and 100% sparged oxygen. Profiles are shown up to 24 h, and are the mean of
303 two independent runs. Oxygen concentration at saturation was ~38 mg/L. The dissolved
304 oxygen for the 0% sparged oxygen drops below detection within 30 min, and the 21%
305 sparged oxygen drops below detection after 4.5 h.
306 To examine the extent of oxygen limitation in the system, 100% oxygen was sparged into
307 the reactor. The profile shows that despite the use of pure oxygen in the gas supplied and
308 the enhanced rate of oxygen supply in the mRDR, the demand for oxygen exceeded the
309 rate of supply under these conditions, and the dissolved oxygen drops below detection
310 after 7 h. From 16 h onwards, as the growth rate of the *S. epidermidis* cells begins to
311 reduce and they enter stationary phase where they consume less oxygen, the dissolved
312 oxygen profile begins to rise to ~35 mg L^{-1} again when oxygen demand diminishes.

313 **Cryoslicing of biofilms cultivated under different oxygen concentrations**
314 Biofilms stained with CTC and DAPI were examined at 40X magnification. Biofilms
315 cultured in oxygen concentrations of 0%, 7% and 21% are displayed in Fig. 4. Thickness
316 measurements are included in Fig. 5. Overall coverage of the coupon surface with
317 biofilm cultivated in 0% oxygen was greater than biofilm grown in 21% oxygen, and a
318 statistical difference is noted ($p < 0.05$). Similar results were reported for biofilm viable

cell counts under these conditions when assessed by colony forming units after the biofilm was removed from the surface of the coupon. These images were acquired with the intention of investigating if gradients in respiratory activity occurred between the biofilms grown in different oxygen conditions. To perform this, any biofilm that could be sliced from the surface of the coupon was used, and thus these pictures give a narrow field of view on the entire biofilm forming on the coupon. However, from what is displayed in these photos, activity gradients are not apparent, with CTC evenly distributed throughout the image.

Antibiotic Susceptibility of biofilm cultivated under different oxygen concentrations

The mRDR can be utilised as a reactor for producing monoculture biofilm, under different oxygen concentrations, for susceptibility testing and thickness measurements. In these experiments rifampicin treatment of biofilm samples grown in the RDR for 48 h in different sparged oxygen conditions were analysed for CFU log reduction after 24 h of treatment. All concentrations of rifampicin tested showed a reduction in viable cells and a reduction in thickness. For the highest concentration of rifampicin tested (2 $\mu g\ ml^{-1}$), biofilm Log CFU cm^{-2} counts in 0% sparged oxygen conditions fell from 8.9 prior to treatment to 7.56 post treatment. In 21% sparged oxygen biofilm samples, Log CFU cm^{-2} counts fell from 8.57 prior to treatment to 7.02 post treatment.

Discussion

Commercial Rotating Disk Reactor (cRDR)

The majority of biofilm reactors available are designed for either reproduction of biofilm, or for visualising the adherence of cells (McClean *et al*., 2004). The RDR is a novel system, and is shown here to be suitable for producing monoculture staphylococcal biofilm for a variety of investigations. Like other commercial bioreactors, the RDR can be used for a variety of purposes, and with the additional applications of cryoembedding and cryoslicing, can produce six biofilm samples adhered to removable coupons that can be quantified or sectioned for microscopy. The RDR can also be used to harvest cells for RNA extraction, which can enable detailed analysis of genetic changes in biofilms in response to imposed environmental conditions as discussed previously (Cotter *et al*., 2009). For this work the authors found the RDR easier to modify than the CDC reactor, and preferable to glass flow cells as biofilm was easier to extract and quantify.

The RDR is completely autoclavable and therefore allows for cultivation of pure culture biofilms on easily removable coupons for ex situ analysis. To enable assessment of viable cells, biofilm was removed by sonication. The design of the RDR disk, which holds the coupons on place, allows contact between the nutrient media and both sides of the coupon. Scraping both the top and bottom of coupons showed no increase in biofilm CFUs compared to scraping the top only, and we conclude that the biofilm forming on the bottom of the coupons is minimal. This may be due to the design of the disk, in which the bottom of the coupon is exposed to the media, but is not flush with the bottom of the rotating disk. This may play a role in reducing the biofilm formation on the

underside of the coupon. This discovery resulted in only the top surface of the coupon being taken into consideration in quantifying biofilm CFU cm^{-2}.

Pumping nitrogen gas into the headspace of a cRDR showed greater biofilm adherence at the interface between the vortex and the disk. A thick, wide band of biofilm that accumulated at this intersection (not shown) is accounted for by the sideways rocking motion that the disk experiences when it rotates at higher speeds. This accumulation band did not correspond with the positioning of the coupon surfaces, and had no bearing on the CFU counts reported.

Comparison of batch and continuous RDR operations must take into account the change in liquid volume due to the influence of the vortex. The positioning of the effluent port (fig. 1), which is closed for batch operations, can become a factor with a larger vortex. In continuous operations the feed volume that needs to be added prior to the liquid volume reaching the height of the effluent port, thus creating continuous conditions, will vary depending on the speed of the disks rotation. This speed differential dictates the height reached by the vortex, and is an important consideration for future work using this reactor.

The rotational speed of the disk had little observed effect on the volumetric mass transfer coefficients of oxygen (k_La) values in the cRDR as increasing the rotational speed of the disk does not alter the size of the interface between the vortex and reactor headspace. In contrast, there is a significant effect of rotational speed on k_La values in the mRDR where the sparger acts as a baffle and the mixing is enhanced. Rotational speed also influences shear at the liquid biofilm interface. The effect of shear on biofilm structure is well documented in different systems (Stoodley et al., 1999a, 1999b; Tsai, 2005). Although

387 the quantification of biofilm structure was not an objective of the present work, the RDR
388 can be expected to be a suitable system for the study of the effect of shear on biofilm
389 structure. Greater rotational speed increases oxygen transfer rate and reduces biofilm
390 formation; these results correlate with previously published data identifying that higher
391 oxygen levels reduce biofilm formation via repression of the *icaADBC* operon in *S.*
392 *epidermidis*, which controls production of the exopolysaccharide PIA (Cotter *et al*.,
393 2009). However, choice of rotational speed in laboratory rotating disk reactors should be
394 selected based on other practical constrains such as the prevention of an excessive vortex
395 which may result in hydrodynamic conditions adjacent to the biofilm resulting in possible
396 detachment.
397
398 **Modified Rotating Disk Reactor (mRDR)**
399 The modifications to the RDR methodology used a constant stream of blended gas
400 through a sparger to accurately control oxygen concentrations within the reactor and
401 media. An important advantage of the RDR is that, unlike flow cells, the hydrodynamic
402 conditions in the bulk liquid surrounding the biofilm do not vary significantly during the
403 course of biofilm development. The presence of the sparger caused the vortex to migrate
404 to the free surface of the media, and though a shallow vortex was still present, it had no
405 interaction with the surface of the disk. With the vortex all but eliminated, the difference
406 in the liquid volume between batch and continuous operations was also negligible, with a
407 volume of 180 ml remaining constant after the initiation of feed. The sparger was
408 designed to add oxygen to the liquid at a higher rate than in the cRDR, moreover it served
409 to eliminate this vortex-disk interaction and distribute the biofilm more evenly across the

surface of the disk. Direct sparging into the media increased the mass transfer of the gas into the liquid by 10 fold compared to the cRDR, and biofilm CFUs were increased further when nitrogen was sparged into mRDR compared to the headspace being pumped with nitrogen in the cRDR. Results in table 2 show a moderate increase in the k_La with increased rotational speed in the cRDR, as the vortex size determines the rate of oxygen transfer from the headspace into the liquid. In contrast, increased rotational speed has a significant positive effect on mass transfer in the mRDR where the k_La is determined primarily by the dispersion of bubbles from the sparged gas. Thus, the potential for strict regulation of environmental conditions and better mass transfer of oxygen in the mRDR indicate the obvious advantages in using this reactor for the purposes of growing biofilm under defined conditions. When comparing the mRDR k_La values with a stirred tank bioreactor, the deficiency in mixing within the mRDR is obvious. But for overall convenience in extracting biofilm for a range of purposes, the system is an important option for *in vitro* studies and could be used in future for similar aerobic and anaerobic investigations using a range of medically or environmentally relevant bacteria. The cells adhering to the coupons still undergo the same shear forces irrespective of the action of the sparger.

The most important and commonly used coupon-based biofilm production system alternative to the RDR is the CDC reactor, also commercially available from Biosurface Technologies (Bozeman, Montana, USA). The CDC reactor incorporates 18 coupons which sit into six removable rods (Williams and Bloebaum, 2010). The input of sparged gas is not an option. In the CDC reactor the coupons are static, with a stirrer used to create shear on the surface of the coupons. This lack of environmental control and

433 difference in coupon quantity are some of few differences between the reactors, as both
434 systems are capable of being used to produce monoculture biofilm adhered to coupons
435 for a variety of analyses.
436
437 **Dissolved Oxygen readings from the RDR**
438 Dissolved oxygen in a biofilm system was measured by non-invasive sensor spots, which
439 have, to our knowledge, not previously been used in biofilm reactors. The sensor spots
440 have previously been used in shake flask cultures for both microbial and mammalian cell
441 culture (Puskeiler *et al*., 2005; Naciri *et al*., 2008). There is a large potential for use in
442 quantifying dissolved oxygen in glass biofilm reactors, as our investigations focusing on
443 biofilm formation on the surface of the spots, which could interfere with the fluorescent
444 quenching system, showed that cell adherence was not at a significant level to affect the
445 readings. Cells did adhere to the spots, but the level of adherence was significantly lower
446 than the quantity of biofilm adhering to the coupons on the rotating disk. Dissolved
447 oxygen measurement in the nutrient media in the mRDR demonstrated the poor mixing in
448 the reactor, and the low oxygen transfer rate that still prevailed despite sparging with gas.
449 Dissolved oxygen profiles were created with pure oxygen sparged into the reactor. Even
450 at this high concentration of oxygen, and high flow rate, complete utilisation of oxygen
451 by the *S. epidermidis* cells occurs. It is assumed that these low levels of dissolved
452 oxygen are likely to create an oxygen limited environment, which alters the biofilms
453 susceptibility to antibiotic attack (Borriello *et al*., 2004). The lack of axial flow in the
454 system may be a reason for the profiles that tend towards 0 mg L^{-1}. The possibility of
455 introducing a second sparger and further gas to increase the levels of dissolved oxygen in

456 the media were hampered by the foaming that occurred when gas flow rates greater than
457 0.5 L min^{-1} were used. This may be related to the size of the bubbles of gas sparged into
458 the reactor. Smaller bubbles would enable greater oxygen transfer into the liquid, but
459 overall it must be concluded that the mRDR significantly increased the amount of oxygen
460 that could be transferred into the liquid when compared to the cRDR.
461 Though the 0% and 21% sparged oxygen profiles look identical from 7 h onwards, it is
462 important to note that there is a difference in the two conditions; cultures sparged with
463 0% oxygen are forced to grow anaerobically, whereas cultures sparged with air consume
464 the amount of oxygen at the rated supplied. The sparged oxygen concentration results in
465 the difference in biofilm accumulation, where a statistical difference was previously
466 noted between the 0% and 21% sparged oxygen concentrations (Cotter *et al*., 2009)
467 The majority of staphylococcal strains that form biofilm are facultative anaerobes, though
468 some strict anaerobes have been isolated (Rowlinson *et al*., 2006). Oxygen conditions
469 vary *in vivo*, and differences in the response of staphylococci are noted, particularly in
470 regard to their production of extracellular polysaccharides (McKenney *et al*., 1999).
471 Expression of PIA, a key extracellular polysaccharide produced by Staphylococci,
472 increases under anaerobic conditions (Cramton *et al*., 2001), as oxygen alters the
473 physiology of the cells. These factors must be considered when attempting to understand
474 the utilisation of available oxygen by planktonic cells and the switch to a biofilm
475 phenotype in aerobic and anaerobic conditions.
476
477 **Analysis of biofilm thickness**

The influence of oxygen on biofilm formation, quantified by adherence of viable cells, translated well in thickness measurements of samples that were cryosectioned and viewed at 40X. Data from the slices showed a statistical difference between the 0% and 21% sparged oxygen biofilms, the same conditions that showed a difference in the CFU counts. The thickness data tested may not be as statistically convincingly as the corresponding CFU data. The resultant images are of such a small area that comparisons in thickness measurements, which are taken directly from these images, may not accurately reflect the differences in biofilm coverage on the coupon. However, from the small area imaged the difference in viable cells does translate to a thickness difference.

Gradients of respiratory activity in the biofilm samples were not visible using CTC and DAPI in combination, however these stains have previously been proven to highlight gradients where present in biofilm systems (Heffernan *et al*., 2009). There is no fundamental reason why the RDR could not be used to view gradients in oxygen or nutrients in a biofilm, if present, provided the correct staining methods are employed. This could result in the RDR coupon system being a convenient way of examining biofilms for gradients prior to removing the entire biofilm for mRNA extraction. It has been shown that zones of DNA synthetic activity and oxygen respiration correspond in *S. epidermidis* biofilms (Rani *et al*., 2007), which could lead to differing mRNA expression levels depending on the where in the biofilm the cells originated from. If no gradients are detected, such as in this case where homogeneity in respiratory activity is present, it eliminates the weakness of using whole biofilm populations to execute mRNA expression profiling.

Antibiotic susceptibility

Biofilms of *S. epidermidis* cultivated on mRDR coupons were analysed for susceptibility to rifampicin. At the highest concentration of rifampicin tested (2 μg ml^{-1}) a large quantity of biofilm remained, and the antibiotic failed to eradicate ~84% of the viable cells in both the 0% and 21% sparged oxygen biofilm samples. The statistically insignificant biofilm susceptibility ($p = 0.18$ by performed by a two way analysis of variance (ANOVA)) under different oxygen concentrations measured here is consistent with the data of Norden and Shaffer (1983), who reported that minimum bactericidal concentrations of rifampicin challenging *S. aureus* were identical under aerobic and anaerobic conditions. Zheng and Stewart (2004) found similar results, but both studies concentrated on the atmospheric oxygen conditions that the rifampicin treatment was performed in. This is the first time biofilm cultured in conditions where the sparged oxygen into the media is controlled. The biofilm is cultivated in a defined sparged oxygen concentration, and rifampicin treatment also takes place in the same oxygen conditions.

The method of antibiotic treatment of biofilm involved washing the antibiotic from the coupon prior to slicing or quantification of viable cells. For the subsequent thickness measurements taken from cryosectioned slices, it is hypothesised that this essential washing step may have removed the dead cells on the top layer of the biofilm, which may account for the erratic nature of the thickness measurements, which show no statistical differences.

522 The potential use of additional fluorescent markers prior to cryoslicing, or susceptibility
523 testing with different or combinations of antibiotics, enable further analysis to be
524 performed using biofilm cultivated on RDR coupons.
525
526 **Conclusion**
527 The commercial RDR is used to grow monoculture biofilm, and has been modified to
528 allow greater control of oxygen levels in the nutrient media. The addition of a sparger,
529 which allowed greater mass transfer of oxygen into the media, also enforced a baffle
530 effect on the system which reduced the vortex caused by the rotation of the disk. Results
531 from the mRDR showed oxygen concentration plays an important role in the quantity of
532 *S. epidermidis* biofilm forming on the removable coupons.
533 The versatility of the RDR and the modifications made to it in this work identify it as a
534 reactor with potential for cultivating pure culture biofilm in defined, controlled
535 conditions. The range of ex-situ analysis described herein, including biofilm and
536 planktonic CFUs, with staining and cryoslicing of biofilm, are performed on biofilm
537 cultivated under defined oxygen concentrations, indicating the potential for the RDR to
538 become an important, commonly used biofilm reactor.
539 Limitations in the mRDR were observed when an increase in the gas flow rate above 0.5
540 L min^{-1} resulted in foaming of the media, which could wet the inlet or outlet filter and
541 compromise the aseptic environment. Increased agitation speed also has an effect on
542 foaming, and the stability of the disk became compromised above 300 rpm.
543 Antibiotic susceptibility of biofilm cultivated in different oxygen concentrations was also
544 examined, with different concentrations of rifampicin. The concentration of oxygen

sparged into the growth media for the biofilm made no statistical difference to the effectiveness of the antibiotic, concurring with previous results (Zheng and Stewart, 2004).

Modifications to the reactor greatly increase the oxygen transfer rate, and with evidence of the influence that oxygen has on *S. epidermidis* biofilm formation, shows an important improvement in the development of the rotating-disk system. Though higher oxygen transfer rates are desirable, the mRDR facilitates a large range of analysis that can be performed with biofilm grown on its removable coupons and is a potential solution for growing monoculture biofilm under defined oxygen concentrations and performing a multitude of quantification and image-based analysis.

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718 **Figure legends**

719

720 1. Schematics of (a) commercial and (b) modified Rotating-Disk Reactor in
721 continuous mode. Dimensions are recorded in mm.
722 2. Planktonic cells of *S. epidermidis* 1457 taken from a modified RDR sparged with
723 (A) 0% oxygen and (B) 21% oxygen at a flow rate of 0.5 L h^{-1}. Quarter strength
724 media was supplied at the rate of 90 ml h^{-1}. Log CFU ml^{-1} (●) and optical density
725 (660 nm) (○) are the mean of three independent reactors.
726 3. Dissolved oxygen profiles for *S. epidermidis* 1457 in the liquid phase grown in a
727 modified RDR sparged with different oxygen concentrations. Profiles are a mean
728 of two independent runs.
729 4. Overlaid fluorescent images of biofilm cryoslices grown in a modified RDR with
730 sparged gas of different oxygen concentrations. Slices of 5 μm thickness were
731 stained with CTC and DAPI, and imaged at 40X magnification. The coupon was
732 located at the bottom of these images before removal prior to slicing. Bar = 50
733 μm. (A) Biofilm from 0% oxygen environment. (B) Biofilm from 7% oxygen
734 environment. (C) Biofilm from 21% oxygen environment.
735 5. Mean thickness measurements from *S. epidermidis* 1457 biofilm grown in a
736 modified RDR under different sparged oxygen concentrations. Error bars are
737 standard error of the mean of two slices of three biofilms, measured 12 times for
738 thickness.

739

740

741

742 **Table legends**

743

744 1. Summary of some published studies where the commercial RDR was used to
745 produce biofilm.
746 2. Volumetric mass transfer coefficients in the mRDR and cRDR at different
747 rotational speeds.

748

749

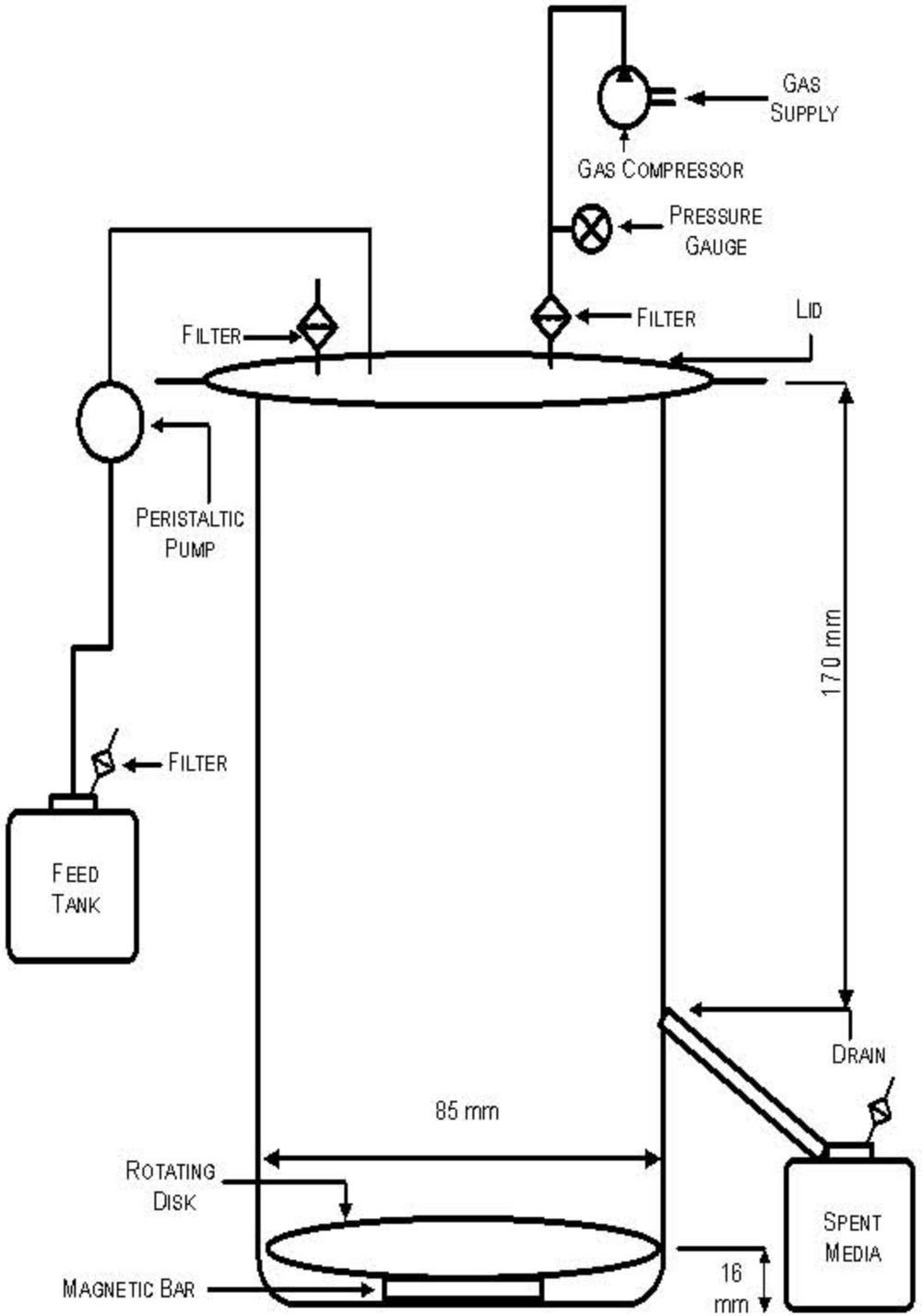
Table 1

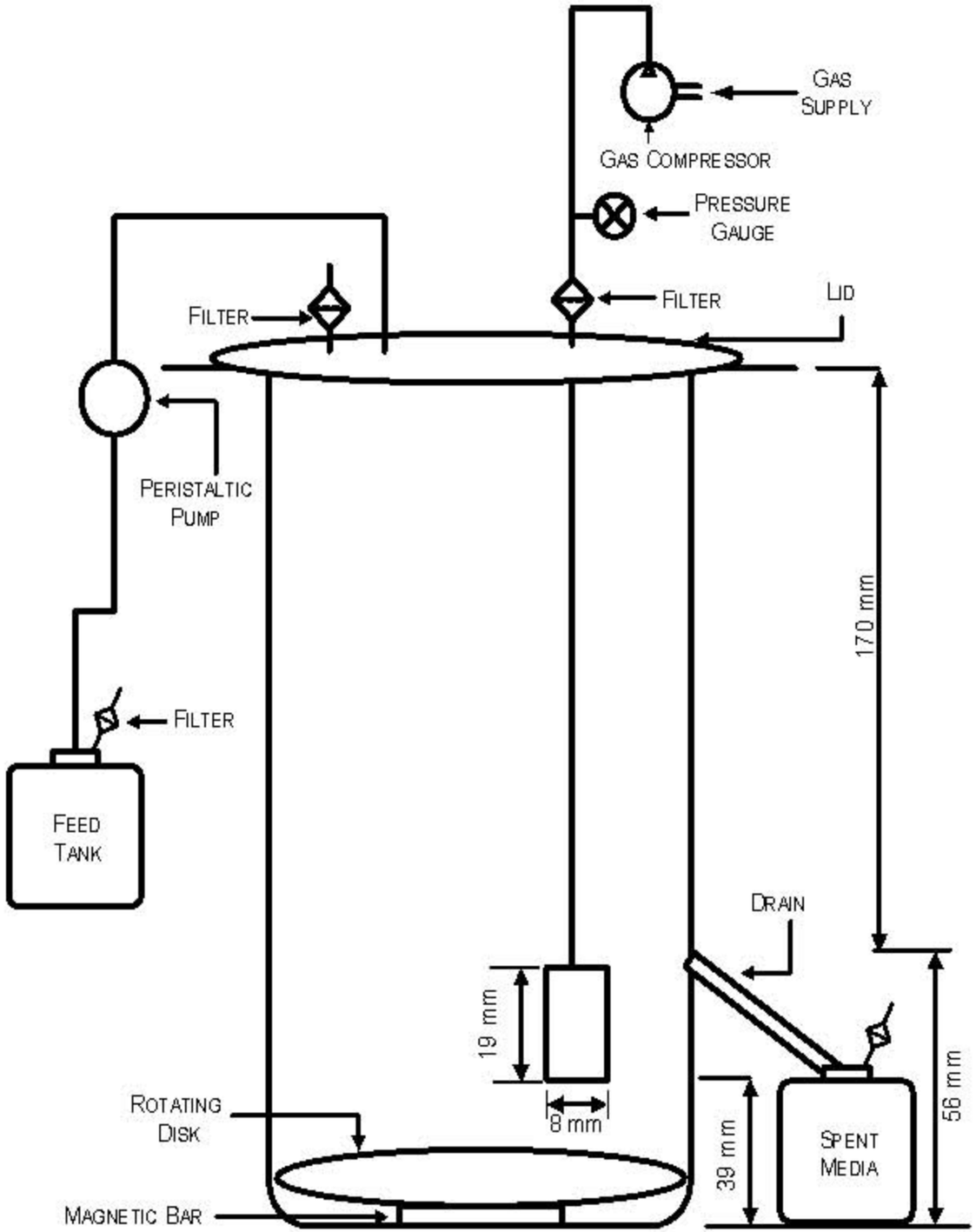
Organism	Operational Mode	RPM	Gas input	Source	Year
Mixed	Continuous	50	None	Khan *et al*.	2010
P. aeruginosa	Continuous	200	None	Garo *et al*.	2007
P. aeruginosa	Continuous	N/A	None	Banin *et al*.	2006
P. aeruginosa	Batch	N/A	None	Boles *et al*.	2004
Streptococcus mutans	Continuous	100	CO_2 filled headspace	Vinogradov *et al*.	2004
P. aeruginosa	Continuous	N/A	None	Teitzel & Parsek	2003
S. aureus	Continuous	N/A	None	Yarwood *et al*.	2003
P. aeruginosa	Continuous	N/A	None	Singh *et al*.	2002
P. aeruginosa	Continuous	N/A	None	Hentzer *et al*.	2001
P. aeruginosa	Continuous	N/A	N/A	Zelver *et al*.	1999
Mixed	Continuous	N/A	N_2 into headspace[†]	Zelver *et al*.	1999

*Note: In this paper the nitrogen is sparged into the bulk feed medium, creating a positive pressure forcing the gas into the headspace of the reactor.

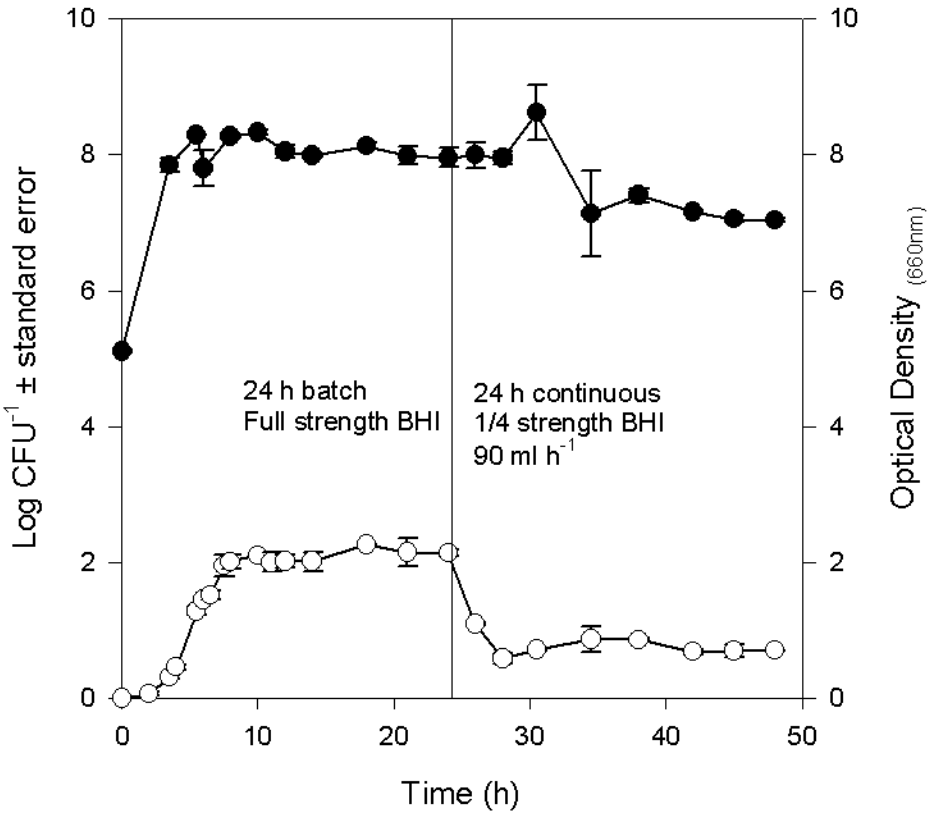
Table 2

Speed (rpm)	k_La (hr^{-1}) mRDR	cRDR
150	0.204	0.0210
200	0.352	0.0323
300	0.950	0.0411

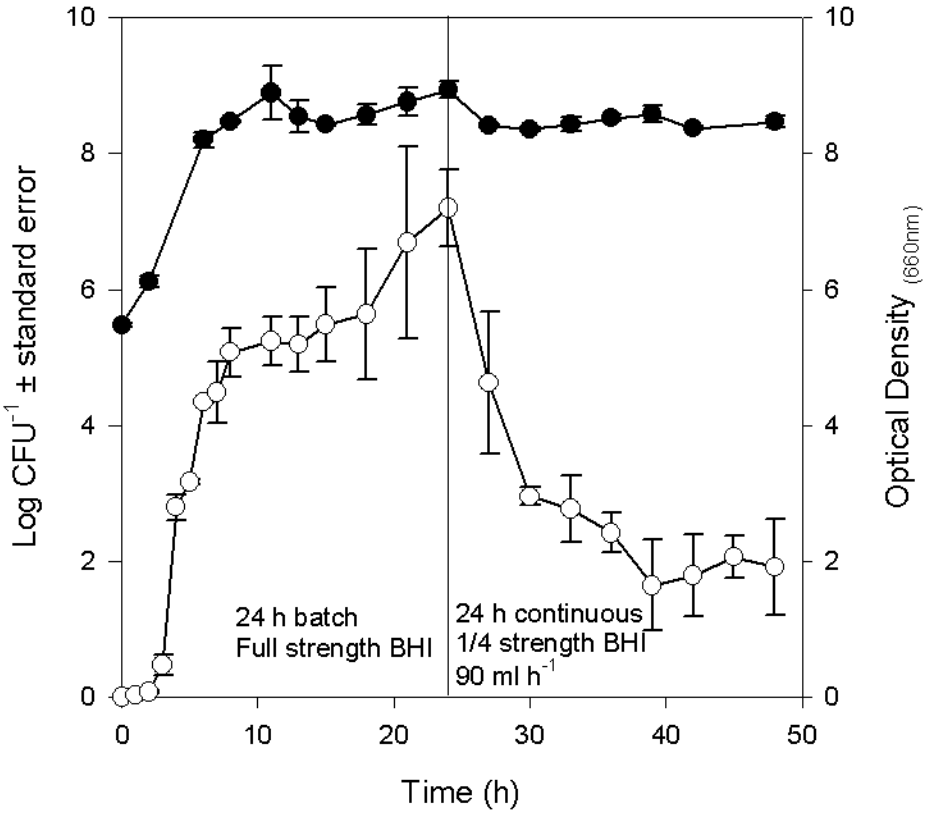

GAS SUPPLY
GAS COMPRESSOR
PRESSURE GAUGE
FILTER
FILTER
LID
PERISTALTIC PUMP
FILTER
FEED TANK
170 mm
DRAIN
85 mm
ROTATING DISK
MAGNETIC BAR
16 mm
SPENT MEDIA


GAS SUPPLY
GAS COMPRESSOR
PRESSURE GAUGE
FILTER
FILTER
LID
PERISTALTIC PUMP
FILTER
FEED TANK
170 mm
DRAIN
19 mm
8 mm
ROTATING DISK
MAGNETIC BAR
39 mm
SPENT MEDIA
56 mm

A


Log CFU^{-1} ± standard error
Optical Density (660nm)
Time (h)
24 h batch
Full strength BHI
24 h continuous
1/4 strength BHI
90 ml h^{-1}
0
2
4
6
8
10
0
10
20
30
40
50

B


Log CFU^{-1} ± standard error
Optical Density (660nm)
Time (h)
24 h batch
Full strength BHI
24 h continuous
1/4 strength BHI
90 ml h^{-1}
0
2
4
6
8
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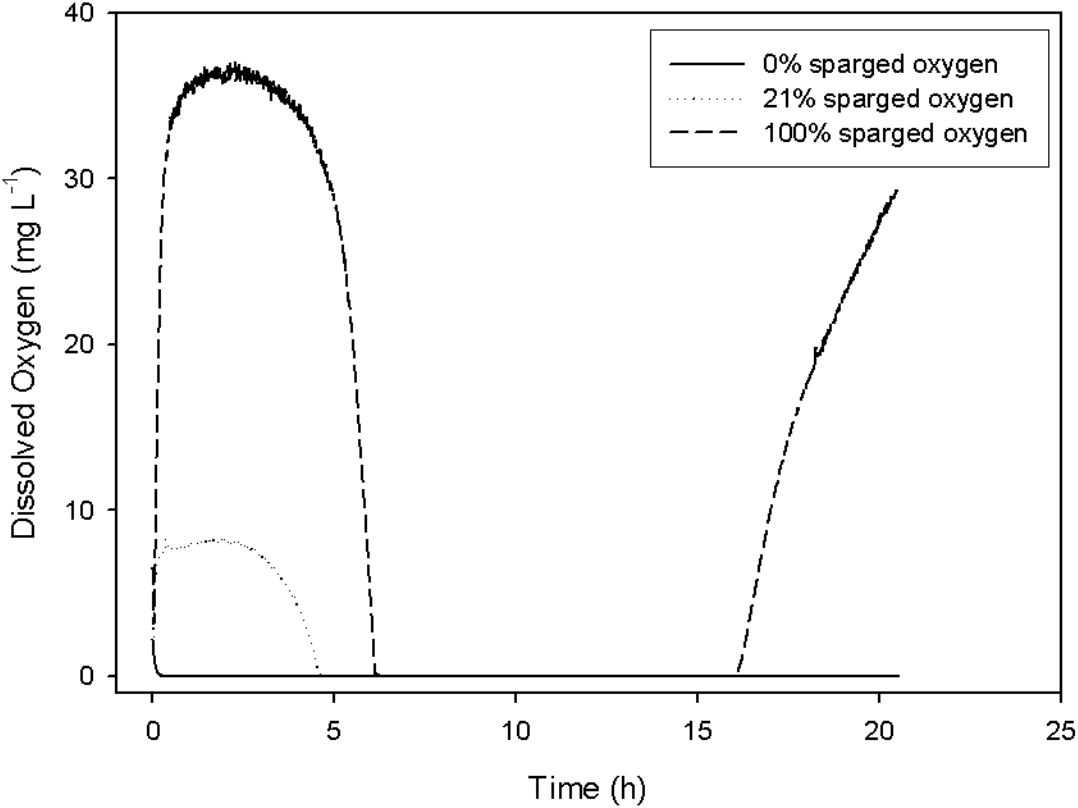
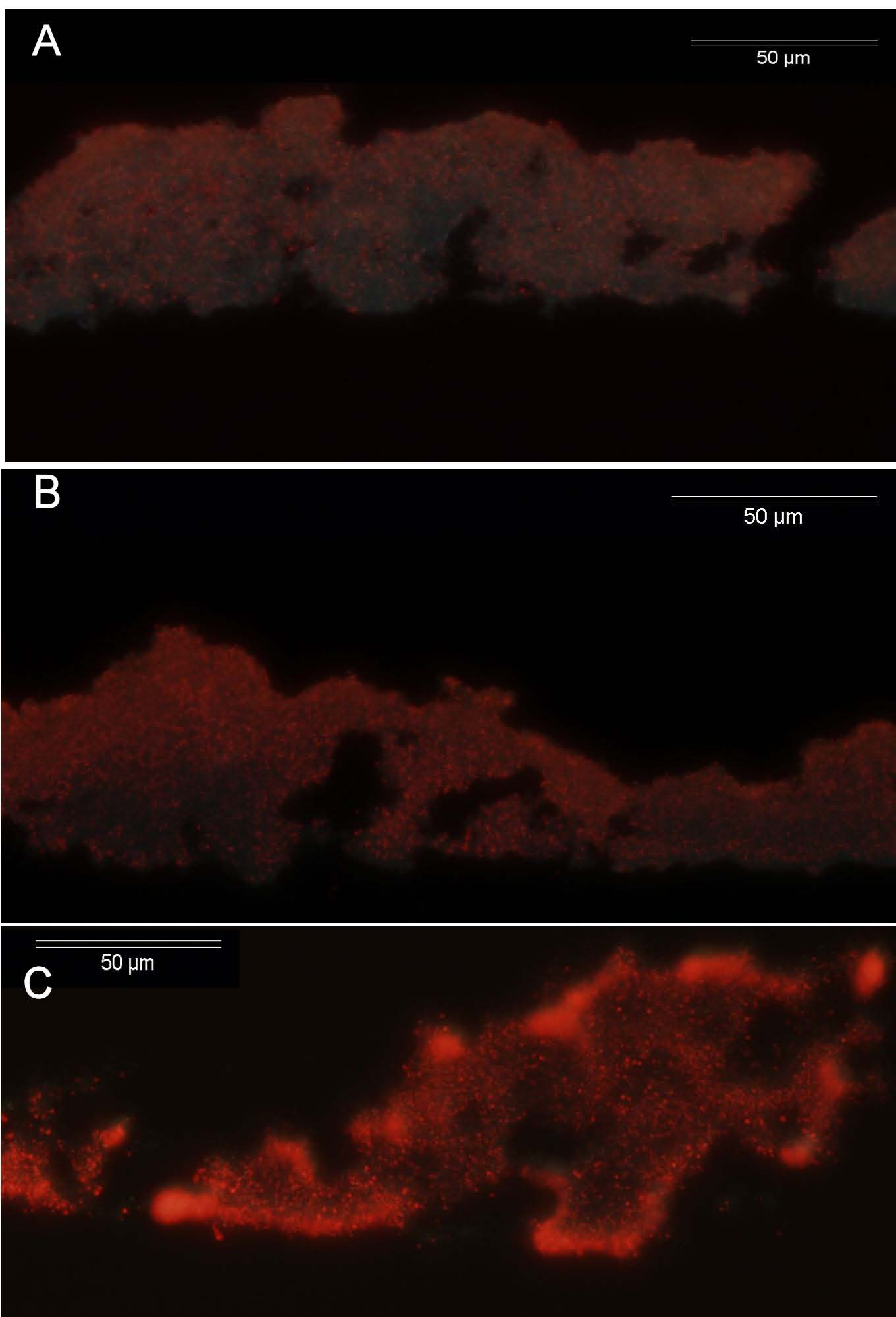
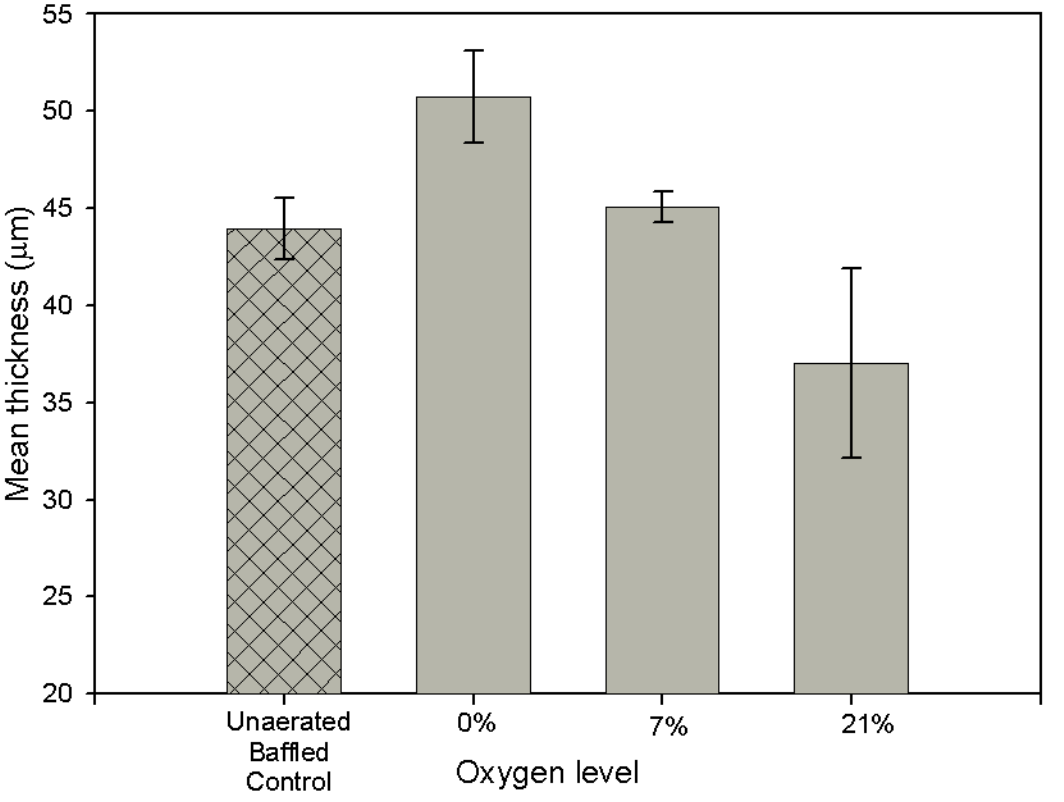

0% sparged oxygen
21% sparged oxygen
100% sparged oxygen
Dissolved Oxygen (mg L-1)
Time (h)
0
10
20
30
40
0
5
10
15
20
25


A
50 µm
B
50 µm
C
50 µm


55
50
45
40
35
30
25
20
Mean thickness (µm)
Unaerated Baffled Control
0%
7%
21%
Oxygen level