Influence of attached growth on the performance of *Pseudomonas fluorescens* grown on fluoroacetate as the sole organic substrate

Running title: Influence of attached growth on P. fluorescens

Barry Heffernan<sup>1</sup>, Cormac D. Murphy<sup>2</sup> and Eoin Casey<sup>1</sup>\*.

<sup>1</sup>UCD School of Chemical and Bioprocess Engineering, Centre for Synthesis and Chemical Biology, and <sup>2</sup>UCD School of Biomedical and Biomolecular Science, 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

#### 1 Abstract

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3 Comparisons between the physiological properties of *Pseudomonas fluorescens* biofilm cells 4 grown in a tubular biofilm reactor and planktonic cells grown in a chemostat were performed. 5 Fluoroacetate was the sole carbon source for all experiments. The performance of cells was 6 assessed using cell cycle kinetics and by determining specific fluoroacetate utilization rates. 7 Cell cycle kinetics were studied by flow cytometry in conjunction with the fluorescent stain 8 propidium iodide. Determination of the DNA content of planktonic and biofilm cultures 9 showed little difference between the two modes of growth. Cultures with comparable specific 10 glycolate utilization rates had similar percentage of cells in the B phase of the cell cycle 11 indicating similar growth rates. Specific fluoroacetate utilization rates showed the 12 performance of planktonic cells to be superior to biofilm cells with more fluoroacetate 13 utilized per cell at similar specific fluoroacetate loading rates. A consequence of this 14 decreased biofilm performance was the accumulation of glycolate in the effluent of biofilm 15 cultures. This accumulation of glycolate was not observed in the effluent of planktonic 16 cultures. Spatial stratification of oxygen within the biofilm was identified as a possible explanation for the overflow metabolism of glycolate and the decreased performance of the 17 18 biofilm cells.

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## 26 Introduction

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28 There is a general consensus that the adhesion of microbes to a surface influences bacterial 29 metabolism, however the experimental results are often contradictory (34). Some studies have 30 compared the physiological status of biofilm and planktonic cells by determining their growth 31 rates, with some (2, 9) reporting increased biofilm growth rates in comparison to planktonic 32 growth rates, while others (1) have reported the opposite. Other researchers have compared 33 the influence of adhesion on biofilm metabolic activity in comparison with planktonic activity 34 and as with growth rate, conflicting observations have been reported (16, 31, 11). The 35 objective of the present study was to characterise planktonic cells grown in a chemostat and 36 biofilm cells grown in a tubular biofilm reactor (TBR) and compare their performance with 37 respect to the degradation of a model xenobiotic compound fluoroacetate.

38 Halogenated compounds are extensively used in many applications (refrigeration, 39 lubricants, pharmaceuticals, insecticides and herbicides) and can be considered significant 40 environmental contaminants. The biodegradation of many chlorinated compounds has been 41 widely reported (5, 26, 28). However, considering the increased use of organofluorine 42 compounds in the past 60 years there is limited information on their degradation (17). 43 Currently a large fraction of wastewater streams containing fluorinated compounds are 44 incinerated (12). Improved biological waste treatment processes require a deeper 45 understanding of microbial degradation of fluorinated compounds.

Some previous studies have focused on the biodegradation of fluorinated aromatic compounds using biofilm reactors (3, 10); however, there have been no studies on the degradation of fluorinated aliphatic compounds in biofilm reactors. Thus sodium fluoroacetate was chosen as the model xenobiotic to study the efficiency of aliphatic organofluorine degradation in biofilms. It was the first naturally occurring fluorinated

51 compound to be isolated, from the South African shrub *Dichapetalum cymosum* (23). 52 Fluoroacetate is highly toxic to mammals and has found extensive use as a vertebrate 53 pesticide, particularly in Australia and New Zealand. A number of studies have focused on the 54 isolation and identification of microbial soil isolates with the ability to degrade fluoroacetate 55 (14, 33, 35), and other studies have focused on the mechanism of defluorination (13, 15, 21). 56 However, there has been no research on the degradation of fluoroacetate by biofilm cultures.

57 Biofilm systems appear ideal for the degradation of xenobiotics considering the many 58 reported advantages they have over planktonic cultures. Most microorganisms that have the 59 ability to degrade xenobiotic compounds have comparatively slow growth rates and biofilm 60 reactors allow the enrichment of these microorganisms independent of hydraulic retention 61 time (36). It has been shown in numerous studies that biofilms are less susceptible to changes 62 in environmental conditions such as temperature, pH, metabolic products and toxic substances 63 than suspended bacteria (8, 25, 27, 36). The high cell concentrations that can be achieved in 64 biofilm systems in combination with high volumetric flow rates could potentially result in 65 high volumetric productivities without the risk of cell washout.

66 The species *Pseudomonas fluorescens* has been extensively studied and commonly 67 exists as biofilm in natural environments and is ubiquitous in industrial environments (6, 29, 68 30). The specific strain used here P. fluorescens DSM 8314, was previously isolated from a 69 soil sample in Western Australia, and in a study with 23 other microbial soil isolates was 70 shown to be the most efficient degrader of fluoroacetate, when fluoroacetate was the sole 71 carbon source (6, 29, 30, 37). The effect of the environmental factors, pH and temperature, on 72 the biodefluorination of fluoroacetate by P. fluorescens was also determined (38); however, at 73 present there are no reported planktonic growth kinetics established for this strain nor has it 74 previously been grown as a biofilm. In this context a tubular biofilm reactor (TBR) was 75 employed to investigate the degradation of fluoroacetate by a *P. fluorescens* biofilm, in

76	conjunction with chemostat studies, which were conducted to determine the efficiency of
77	planktonic degradation of the substrate. Specific utilization rates, flow cytometry and
78	fluorescent microscopy were employed to compare the performance and physiological status
79	of biofilm and planktonic cells grown with fluoroacetate as the sole organic substrate.
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### 101 Materials and Methods

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#### 103 Medium and culture conditions.

104 *Pseudomonas fluorescens* (DSM 8341) was obtained from the German Collection of 105 Microorganisms and Cell Cultures (DSMZ, Germany). Brunners minimal medium (DSMZ, 106 medium 457) supplemented with sodium fluoroacetate (Sigma, UK) as the sole organic 107 substrate was the growth medium for all experiments with the exception of the 50 mM 108 biofilm experiments. In these experiments the concentration of  $KH_2PO_4$  was increased from 109 1.52 to 15.2 g/l in order to provide greater buffering capacity.

#### 110 **Biofilm reactor**

The biofilm was grown in the lumen of a silicone tube (Alteil<sup>TM</sup>, UK) referred to as a tubular 111 112 biofilm reactor (TBR), which is schematically presented in Fig 1. The nominal inner 113 dimension of the silicone tubing was 3 mm and the wall thickness was 1 mm. The system 114 consisted of a medium reservoir, peristaltic pump, a glass flow break, four 40 cm sections of 115 silicone tubing separated by 5 sample ports, a glass flow break and a spent medium reservoir. 116 This reactor configuration allowed the determination of local metabolite concentrations and 117 biofilm characteristics and thus each section can be considered an independent reactor. Four 118 separate TBR experiments were performed, with concentrations of 10, 20 and 50 mM 119 fluoroacetate in the medium feed; the 20 mM experiment was repeated. Thus 16 different 120 specific fluoroacetate loading rates were examined (four reactors with four sections each). 121 Cells were grown for 24 h at 30 °C in batch culture prior to reactor inoculation to ensure the 122 cells were in the exponential phase of growth. The 24 h culture was adjusted to a turbidity of 123 approximately 0.1 at 660 nm in phosphate-buffered saline (PBS), mixed with 10 ml of 124 medium, and inoculated into the reactor. Following inoculation the system was operated in static mode for approximately 48 h at 30 °C after which time the flow of medium was initiated 125

126 at a flow rate of 6 ml/h (velocity 0.023 cm/s) and maintained throughout the experiments. The 127 dilution rate was approximately 2.5 times greater than the maximum planktonic growth rate; 128 this was chosen to ensure the washout of planktonic cells and encourage biofilm formation. 129 The reactor was sampled daily from sample port 5 and from all five ports 24 h prior to biofilm 130 harvesting. The following parameters were analysed: optical density, fluoroacetate, free 131 fluoride ion, glycolate, DNA content of cells and CFUs. The biofilm was allowed to develop 132 for approximately 300 h, at which time the bioreactor was disassembled and the biofilm was 133 harvested for analysis.

# 134 **Biofilm harvesting**

Prior to biofilm harvesting the TBR was drained of bulk liquid to prevent any interference of detached cells. Biofilm cells were harvested from the inside of the tubing which was sectioned into 5 cm divisions with thickness, dry weight, DNA content of cells, and CFUs being measured. Biofilm was removed from the lumen of the tubing by pinching the outer surface of the tubing, the lumen was washed with (PBS) and the disrupted biofilm was harvested.

## 141 Chemostat trials

142 Chemostat studies were performed in duplicate in a 3 1 bioreactor (Applikon, The 143 Netherlands) with a working volume of 1.5 l and the performance of planktonic cells was 144 assessed at four fluoroacetate loading rates representing growth rates between 45 and 80 % of 145 the maximum growth rate. The reactor was equipped with controls for agitation, pH, 146 temperature and dissolved oxygen. For pH adjustment 1 M NaOH was used. The inlet gas 147 flow rate was maintained at a constant rate of 1.5 l/min by use of a mass flow controller. 148 Dissolved oxygen was controlled by agitation and maintained at or above 40 % air saturation 149 throughout the experiments. Temperature was maintained at 30 °C throughout the 150 experiments. The reactor was inoculated with 15 ml of 24 h old culture adjusted to an optical density of 0.1 at 660 nm. The reactor was sampled periodically and the following parameters were analysed: optical density, fluoroacetate, free fluoride ion, glycolate, DNA content of cells, dry weight and CFUs. The reactor was operated under batch conditions during the exponential phase of growth, at the onset of the stationary phase of growth the reactor was switched to continuous operation. The feed flow rate was measured by recording the mass of liquid effluent over a specified period of time using a balance (Mettler Toledo, USA). The initial fluoroacetate concentration during the batch phases of growth was 20 mM.

#### 158 Batch growth trials

Planktonic growth rates were determined for initial fluoroacetate concentrations of 10, 20 and 50 mM in 250 ml conical flasks incubated at 30 °C with shaking at 150 rpm. The flasks were operated with a working volume of 50 ml and inoculated with a 0.5 ml of a 24 h old culture adjusted to an optical density of 0.1 at 660 nm with PBS. The flasks were sampled periodically and analysed for optical density and free fluoride ion.

#### 164 Anaerobic batch culture experiments

165 Anaerobic batch culture experiments were performed in triplicate with 20 mM of 166 fluoroacetate or 20 mM of glycolate respectively as the sole carbon source in 100 ml Schott 167 Duran bottles with a working volume of 30 ml. An anaerobic environment was maintained by 168 continuous subsurface sparging of nitrogen gas at a flow rate of 0.5 ml/min. This flow rate 169 was found to be sufficient to ensure an anaerobic environment while also avoiding liquid 170 losses due to evaporation over the 8 h duration of the experiment. The Duran bottles were 171 inoculated by harvesting 30 ml of a 24 h old culture grown on fluoroacetate; the harvested 172 cells were then resuspended in 1 ml of fresh media prior to inoculation. The bottles were 173 incubated at 30 °C with shaking at 150 rpm for 8 h at which time they were sampled and 174 analysed for optical density, dissolved oxygen and metabolite concentrations. After the 8 h 175 incubation period the dissolved oxygen concentration was measured in both the anaerobic and aerobic experiments. There was no dissolved oxygen detected in any of the anaerobic
experiments while the dissolved oxygen concentrations in the aerobic control bottles were
found to be above 40 % air saturation after the 8 h incubation period.

### 179 **Epifluorescence microscopy**

180 A 5 cm section of tubing containing biofilm was washed with PBS (1 ml) and stained with 5-181 cyano-2,3-ditoyl tetrazolium chloride (CTC; 400 µg/ml), for 2 h at 30 °C. The tubing was 182 drained, washed with PBS (1 ml) and stained with 4'-6-diamidino-2-phenylindole (DAPI; 100 183 µg/ml), for 30 min at ambient temperature. The tubing was washed with PBS and embedded 184 with 5 ml of O.C.T. histological cryoembedding medium (Tissue-TEK O.C.T. compound), 185 which was gently injected into the tubing. The tubing was then placed in an embedding 186 chamber for 10 min to solidify the O.C.T. and samples were stored at – 80 °C until sectioning. 187 The embedded biofilm was sectioned (10 µm cross sections), after removing the biofilm from 188 the silicone tubing, using a Microm HM 550 cryostat (Microm, Walldorf, Germany). The 189 sections were placed on glass slides and examined with an Olympus BX51 epifluorescence 190 microscope using a 4-x objective. Photographs were obtained with an Olympus DP70 digital 191 camera. The exposure time when acquiring CTC images was 500 ms, and 50 ms when 192 acquiring DAPI images.

# 193 Thickness measurements and image analysis

Biofilm thickness was analysed by Able Image Analyser software (Mu Labs, Slovenia). Thickness was measured as the distance from the membrane to the biofilm liquid interface. From each section of the reactor six phase contrast images were analysed for thickness with 25 measurements taken from each image. These measurements were then averaged to give a final thickness. Depth profiles of respiratory activity (CTC) and biomass (DAPI) were measured perpendicular to the membrane in overlapped CTC and DAPI images acquired at the same point. The interface between the biofilm and the membrane was set at zero. Fifteen 201 individual profiles were performed in six different images from each section. Three 202 representative profiles were then averaged to produce a final radial profile of respiratory 203 activity and biomass for each section

204 **Dry weight** 

Biofilm dry weight was determined by drying a 5 cm section of tubing at 60 °C for 48 h, at which point a constant weight was achieved. The tubing was immersed in warm water to hydrate the biofilm, and the biofilm was removed by pinching the silicone tubing, which was then washed vigorously with water to remove any residual biofilm. The silicone tubing was dried for a further 48 h and reweighed. The weight of the biofilm was calculated by subtracting the dry weight of the empty tubing from the weight of the tubing plus biofilm.

### 211 Specific utilization rate

212 The specific utilization rate (q) is defined as the concentration of substrate degraded per unit 213 time per CFU. The utilization of a carbon/energy substrate is separated into two fluxes (Fig 2) corresponding to consumption of the substrate for incorporation into biomass (q  $_{\rm an})$  and 214 215 utilization of substrate for energy which can be further subdivided into the energy required for 216 growth  $(q_{en})$  and energy required for maintenance  $(q_m)$ . In some cases it is possible that there 217 is a rate-limiting step in the overall catabolic pathways, which leads to the accumulation of an 218 intermediate metabolite and is described here as overflow metabolism (Om). Fluoroacetate 219 was the sole organic substrate available to the cells and is initially degraded by the enzyme 220 fluoroacetate dehalogenase (Fig 3), yielding free fluoride ion and glycolate; glycolate is then 221 utilized as the carbon/energy source. Figure 3 shows that carbon is conserved in the 222 fluoroacetate dehalogenase reaction thus there are two specific utilization rates q f, which is 223 the specific utilization rate of fluoroacetate, which is a single enzymatic reaction, and results 224 in the production of glycolate, and q g, the specific utilization rate of glycolate consumed for 225 the production of cell material  $(q_{an})$  and ATP generation  $(q_{en} \text{ and } q_{m})$ . Unconsumed

226 glycolate (Om) is transported across the cell membrane into the bulk liquid. In order to 227 compare  $q_f$  and  $q_g$  directly specific utilization rates were calculated in mM carbon.

#### 228 Flow cytometry

229 Cells were harvested from biofilm and planktonic cells as described, diluted to a concentration of approximately  $5 \times 10^6$  cells per ml and washed three times in PBS. This was 230 231 performed by re-suspending the biofilm in PBS, centrifuging for 5 min at 14,000 rpm and 232 discarding the supernatant. The washed cells were suspended in 0.1 ml of PBS and 1 ml of 233 ice-cold 70% ethanol. The cells were kept at 4 °C for 24 h to fix the cells prior to transfer to – 234 20 °C for storage. Prior to analysis the cells were separated from the ethanol by centrifugation 235 and re-suspended in 1 ml of PBS containing propidium iodide (80  $\mu$ M). Cells were then 236 analysed for DNA content using a Dako Cyan ADP flow cytometer (Dako, Glostrup, 237 Denmark) and histograms showing fluorescence intensity against cell number were generated 238 (Fig 4). The different phases of cell cycle (B, C and D) were then determined from these 239 histograms using Multicycle software (Phoenix Flow Systems, USA).

## 240 Fluoroacetate, free fluoride ion and glycolate analysis

241 Free fluoride ion was measured using an ion selective fluoride combination electrode 242 (Thermo Orion model 290). Fluoroacetate concentration was determined by fluorine-19 nuclear magnetic resonance (<sup>19</sup>F NMR) spectroscopy. Samples were prepared by mixing 0.6 243 244 ml of culture fluid with 0.2 ml D<sub>2</sub>O (added to provide a lock signal) and analysis was 245 performed using a Varian 400 MHz spectrometer. The known free fluoride concentration in 246 each effluent sample was used as an internal standard. The ratio of free fluoride ion signal (-247 120 ppm) to fluoroacetate signal (- 215 ppm) was used to calculate fluoroacetate 248 concentration. Glycolate was measured by the method described by (20).

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- 251 Results
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## 253 Biofilm formation, growth and structural characteristics

254 The TBR system was employed as a method to characterise *P. fluorescens* biofilms grown at 255 varying carbon loading rates. Carbon loading rate is defined here as the total carbon available 256 in a section of tubing, it includes both the carbon in the form of fluoroacetate and the carbon 257 in the form of glycolate. It has been shown that fluid velocity is a critical parameter in 258 determining biofilm adhesive strength for *P. fluorescens* biofilms (4), accordingly in order to 259 maintain a constant velocity in all experiments carbon loading rates were varied by altering 260 the fluoroacetate inlet medium concentration and not the dilution rate. Experiments were 261 performed with initial fluoroacetate concentrations of 10, 20 and 50 mM in the medium feed. 262 Visual observation of initial biofilm formation occurred between 116 and 160 h after reactor 263 inoculation. Biofilm appearance, as indicated by colony formation on the lumen of the 264 silicone tube, depended on the carbon loading rate; in experiments employing 10 and 20 mM 265 fluoroacetate, where carbon loading rates were between 0 and 0.190 mM/h, biofilm growth 266 was apparent in a shorter period of time than in experiments with 50 mM fluoroacetate, where 267 carbon loading rates were between 0.300 and 0.510 mM/h. Steady state, as indicated by 268 fluoroacetate, glycolate and free fluoride ion concentrations in the effluent, was achieved 269 earlier in the experiments with lower carbon loading rates. In the reactors with initial carbon 270 loading rates of less than 0.190 mM/h, complete carbon utilization had occurred by the time 271 steady state had been reached. However, in the reactor with carbon loading rates of above 272 0.300 mM/h approximately 3 mM of fluoroacetate and 18 mM of glycolate were still 273 detectable at steady state.

Visual observations of the biofilm formation provides some qualitative information as
to when initial biofilm formation occurs and measured metabolite concentrations throughout

276 the time course of the experiments indicate when steady state conditions have been reached, but these data do not provide any quantitative measurement of biofilm structure or 277 278 performance. Thus, biofilm characteristics of thickness, dry weight, cell number and density 279 were calculated at different sections of the reactor (Fig 1), for different carbon loading rates 280 after biofilm harvesting (Table 1). Cross sectional biofilm thickness was calculated from 281 phase contrast images. These thickness measurements show little variation in the average 282 biofilm thickness for all sections where the carbon-loading rates was above 0.017 mM/h 283 where the average was 116.8  $\mu$ m with a standard deviation around the mean of  $\pm$  22.9  $\mu$ m. An 284 exception to this trend was the section with a carbon loading rate of 0.107 mM/h where the 285 average thickness was 130 µm thicker than that recorded in any other section. The same 286 trends observed for average thickness were observed for dry weight, CFU and density 287 measurements with a few exceptions most notably for the section with a carbon loading rate 288 of 0.107 mM/h. Images taken from the section with this carbon loading rate suggest that the 289 biofilm may have been in the process of sloughing and this could explain the greater thickness 290 and lower density values recorded in this section. The 20 mM reactor experiment was 291 performed in duplicate, and Anova single factor statistical analysis showed that there was no 292 significant difference between biofilm thickness, dry weight, cell number and density values 293 recorded in these two reactors (F= 0.15, F<sub>crit</sub>=4.7 and p=0.69). Biofilm was present in three 294 individual sections of the reactors where the carbon loading rate was 0 mM/h, on account of 295 carbon source depletion and in these sections the presence of biofilm was probably a result of 296 carbon being present during the early stages of the reactor operation.

#### 297 Comparisons between planktonic and biofilm performance

298 Comparisons between biofilm and planktonic cells are difficult to make due to the intrinsic 299 difference between the two modes of growth, such as compositional differences in biomass. 300 Typically, performance is determined by specific utilization rates (q), where q is defined as

301 the concentration of substrate degraded per unit time divided by the dry weight of biomass. 302 However, dry weight measurements do not take into account EPS, which will account for a 303 much higher fraction of biofilm biomass than it will for planktonic biomass. It has been 304 demonstrated that the composition of P. fluorescens B52 extracellular polymeric substances 305 (EPS) is different for biofilm and planktonic cells (18). Thus, in this study q was calculated 306 using CFUs rather than dry weight. CFU measurements are advantageous in this situation as 307 they are a measure of viable cells for both biofilm and planktonic systems. The reactor 308 working volume for the chemostat was 1.5 l while the reactor volume in each section of the 309 TBR was only 0.0028 1. To account for this difference in reactor volumes, specific 310 fluoroacetate loading rates (mM/CFU h), rather than fluoroacetate loading rates (mM/h), were 311 used in conjunction with specific fluoroacetate and glycolate utilization rates to compare the 312 performance of biofilm and planktonic cells (Fig 5). For the range of loading rates 313 investigated the data clearly reflect higher utilization rates for planktonic cells than for biofilm 314 cells. Linear regression analysis of fluoroacetate utilization rates at specific fluoroacetate loading rates between 0 and  $14 \times 10^{-12}$  mM/CFU h show a linear relationship for both biofilm 315  $(R^2=0.91)$  and planktonic cells  $(R^2=0.90)$ . Biofilm fluoroacetate loading rates above  $14 \times 10^{-12}$ 316 317 mM/CFU h were not included in the regression analysis as the performance of planktonic 318 cells was not assessed above this range. The performance of planktonic cells was superior to 319 biofilm cells with a higher dependency of specific fluoroacetate utilization recorded for 320 planktonic cells (slope=0.87) than for biofilm cells (slope=0.57). Specific glycolate utilization 321 rates for planktonic cells were the same as specific fluoroacetate utilization rates, thus the 322 fluoroacetate degradation/glycolate production rate was equal to the rate of glycolate 323 utilization for planktonic cells at all specific fluoroacetate loading rates examined. However, 324 there were significant differences between specific glycolate and specific fluoroacetate utilization rates for biofilm cells at all specific fluoroacetate loading rates above  $2 \times 10^{-12}$ 325

326 mM/CFU h (t-stat=1.43, p=0.091) (based on a one-tailed t-test, assuming unequal variances).
327 A result of this difference between specific fluoroacetate and glycolate utilization rates for
328 biofilm cells was the accumulation of glycolate (overflow metabolism). Thus, the data
329 indicate a rate-limiting step in the utilization of glycolate by biofilm cells that was not
330 observed for planktonic cells.

### 331 **Oxygen limitation**

332 Depth profiles of respiratory activity as indicated by CTC and biomass concentration as 333 indicated by DAPI show spatial stratification of respiratory activity within the biofilm (Fig 6). 334 The region of highest respiratory activity is located adjacent to the biofilm membrane 335 interface where oxygen concentration is highest. Respiratory activity decreases towards the 336 bulk liquid interface while biomass concentration does not, suggesting decreased oxygen 337 availability in this location. While it was not possible to measure the oxygen profiles within 338 the biofilm in this study, the penetration depth of oxygen into a biofilm is governed by a 339 reaction-diffusion interaction. The relative rate of oxygen diffusion and oxygen consumption 340 by the biofilm determines the depth of oxygen penetration, which can be determined theoretically from the following equation (32):  $a = (2D_e S_o / k_o)^{1/2}$ , where *a* is the 341 penetration depth of the reacting solute,  $D_e$  is the effective diffusion coefficient of the solute 342 in the biofilm,  $S_o$  is the solute concentration at the biofilm interface and  $k_o$  is the volumetric 343 344 reaction rate of the solute inside the biofilm. Estimates of each of these parameters were made 345 to allow the calculation of oxygen penetration depth. The effective diffusion coefficient of 346 oxygen in the biofilm at 30 °C was taken as 57% of the diffusion coefficient of pure water at this temperature (32), or  $1.28 \times 10^{-5} \text{ cm}^2 \text{s}^{-1}$ . The concentration of dissolved oxygen at the 347 biofilm membrane interface was taken as 8.0 mg litre<sup>-1</sup>, and the volumetric consumption rate 348 of oxygen was determined experimentally to be 1.64 mg litre<sup>-1</sup> s<sup>-1</sup>. From these estimates the 349 350 calculated depth of oxygen penetration into the biofilm from the membrane was 111  $\mu$ m. The

351 average biofilm thickness in the sections of the reactors where overflow metabolism of glycolate occurred was 118  $\mu$ m ± 21  $\mu$ m. These oxygen depth calculations combined with 352 353 biofilm thickness measurements and the spatial stratification of respiratory activity within the 354 biofilm suggest that oxygen does not completely penetrate into the biofilm. A study by (7) 355 demonstrated that the fluoroacetate dehalogenase enzyme isolated from P. fluorescens does 356 not require the presence of oxygen to degrade fluoroacetate. Thus, a possible explanation for 357 the overflow metabolism of glycolate is oxygen limitation. Anaerobic batch culture 358 experiments were performed to test the hypothesis that oxygen limitation was the reason for 359 the overflow metabolism of glycolate in the TBR. There was no growth, as indicated by 360 optical density measurements, in either the fluoroacetate or glycolate anaerobic bottles after 8 361 h of incubation, demonstrating that oxygen is require for biomass production. However, when 362 the cells were incubated with fluoroacetate under anaerobic conditions  $1.2 \pm 0.01$  mM of 363 glycolate and  $1.4 \pm 0.05$  mM of free fluoride ion were detected in the effluent. Thus, 364 fluoroacetate was degraded in the absence of oxygen but glycolate was not and the data there 365 by supports the possibility that oxygen is the rate limiting step in overall glycolate catabolism. 366 Interestingly, when P. fluorescens was grown aerobically there was greater biomass 367 production when the cells were grown on glycolate as the sole carbon source (O.D  $0.811 \pm$ 368 0.11) than when the sole carbon source was the fluoroacetate (O.D  $0.411 \pm 0.16$ ), the OD at 369 time 0 was 0.150 for all experiments. The cells grown on glycolate utilized more carbon (16.2 370  $\pm$  0.3 mM) than cells grown on fluoroacetate (8.5  $\pm$ 0.45 mM) in the same time period which is 371 surprising considering the cells were adapted to growth on fluoroacetate and not glycolate. As 372 with the shake flask and chemostat experiments, no glycolate was detected in the effluent of 373 the control fluoroacetate aerobic experiment indicating that the fluoroacetate degradation rate 374 was equal to the glycolate utilization rate when oxygen is not limiting.

## 375 Comparisons between the DNA content of planktonic and biofilm cells

376 Bacteria with doubling times between 0.3 and 1 h can have multiple copies of DNA making 377 cell cycle analysis difficult due to overlapping replication cycles. Batch culture planktonic 378 growth trials were performed to determine the doubling times of *P. fluorescens* grown on 10, 379 20 and 50 mM fluoroacetate, and were found to be 8.3, 6.6 and 7.1 hours, respectively. The 380 B-phase of the bacterial cell cycle can be described as the time between cell division and the 381 initiation of a new round of replication; the C-phase is the time between initiation and 382 termination of chromosome replication; and the D-phase is the time between chromosome 383 termination and cell division. The C and D-phases of growth can be considered constants. 384 However, at growth rates of less than 1 doubling per hour the B-phase of bacterial growth is 385 not constant but increases with decreasing growth rate, thus an increase in the percentage of 386 cells in a population in the B-phase can be expected in slowly growing cells (19). The 387 distributions of biofilm and planktonic cells in the various phases of cell cycle (B, C and D) 388 are compared with specific glycolate utilization rates (Fig 7). Cell cycle distributions were 389 compared using specific glycolate utilization rates and not specific fluoroacetate utilization 390 rates, as the former is a measurement of the utilization of substrate for carbon and energy, 391 while the latter is a measurement of a single enzyme activity. Biofilm and planktonic cells 392 that had zero specific glycolate utilization rates (zero carbon loading rates) as expected have a 393 very high percentage of cells in the B phase (approximately 90 %), while there were very few 394 cells in the D phase of cell cycle. On the basis on a one-tailed t-test, assuming unequal 395 variances, the data shows that, for biofilm cells, increased specific glycolate utilization rates from between 0.06 and 2  $\times$  10  $^{-12}$  mM/CFU h to between 4.5 and 8  $\times$  10  $^{-12}$  mM/CFU h 396 397 significantly increases the percentage of cells in the B (t-stat=3.98, p=0.00266). No carbon in 398 the form of either fluoroacetate or glycolate was detected in the effluent of these sections and thus, it is probable that these cells had decreased growth rates, of less than  $0.1 \text{ h}^{-1}$ , as a result 399

400	of carbon limitation. The data suggest that there is no significant difference between the
401	percentage of cells in the B phase of growth for planktonic and biofilm cells, investigated at
402	similar specific glycolate utilization rates between 4 and 8 x $10^{-12}$ mM/CFU h (t-stat=0.25,
403	p=0.403), suggesting that the two populations of cells have similar growth rates between 0.1
404	and 0.12 $h^{-1}$ (based on a one-tailed t-test, assuming unequal variances). There was a
405	statistically significant decrease in the percentage of cells in the B phase of growth when
406	planktonic specific glycolate utilization rates increased from between 4 and 8 x $10^{-12}$
407	mM/CFU h to between 8 and 12 x $10^{-12}$ mM/CFU h (t-stat=1.57, p=0.09) (based on a one-
408	tailed t-test, assuming unequal variances). This decrease in the percentage of cells in the B
409	phase was as expected, as the cells with specific glycolate utilization rates between 8 and 12 x
410	$10^{-12}$ mM/CFU h are utilizing more carbon, have higher growth rates between 0.14 and 0.18 h <sup>-12</sup>
411	<sup>1</sup> and consequently have fewer cells in the B phase of growth as opposed to the cells with
412	specific glycolate utilization rates between 4 and 8 x $10^{-12}$ mM/CFU h where the growth rates
413	were between 0.1 and 0.12 $h^{-1}$ .
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#### 425 Discussion

426 The increasing number of applications that rely on organofluorine compounds has resulted in 427 them becoming universal environmental contaminants (24). However the degradation of 428 fluorinated xenobiotics is poorly understood in comparison to other halogenated compounds, 429 where research has tended to concentrate on chlorinated and brominated pollutants. 430 Biodegradation pathways can result in the accumulation of intermediates that are potentially 431 inhibitory or toxic (22), and because of the importance of diffusion in biofilms the 432 accumulation of such intermediates could potentially affect performance. The biodegradation 433 of the model xenobiotic, fluoroacetate, by P. fluorescens was investigated and comparisons 434 between the performance of planktonic and biofilm cells were made.

435 Here two methods have been used to determine the physiological status and activity of 436 planktonic and biofilm cells. First, the metabolic activity of biofilm and planktonic cells were 437 assessed in terms of specific utilization rates. It was found that the performance of planktonic 438 cells was superior to biofilm cells with planktonic cells mineralizing more fluoroacetate per 439 CFU per hour than the biofilm cells. When the cells were grown under planktonic conditions 440 there was no difference between specific fluoroacetate and specific glycolate utilization rates 441 at any of the specific fluoroacetate loading rates examined (Fig 5). This was not the case for 442 the biofilm cells, and specific glycolate utilization rates were decreased in comparison to their 443 corresponding specific fluoroacetate utilization rates at a number of specific fluoroacetate 444 loading rates. Thus, the major differences between biofilm and planktonic cells were the 445 decreased performance of the biofilm cells in terms of fluoroacetate and glycolate utilization 446 and the overflow metabolism of glycolate.

It has been shown previously by (7) that the fluoroacetate dehalogenase enzyme isolated from *P. fluorescens* does not require the presence of oxygen to degrade fluoroacetate and in this study it has been demonstrated that the utilization of glycolate via aerobic

450 respiration requires oxygen as a terminal electron acceptor. Thus, oxygen limitation within the 451 biofilm offers an explanation for the overflow metabolism of glycolate. Spatial stratification 452 of respiratory activity, as indicated by CTC, supports the possibility of oxygen limitation 453 within the biofilm. Respiratory activity is highest at the biofilm/membrane-interface and 454 decreases towards the biofilm/bulk liquid interface (Fig 6). This spatial stratification of 455 nutrients within the biofilm is a major difference between biofilm and planktonic cells. For 456 planktonic cells grown in the chemostat system the dissolved oxygen concentration was 457 controlled at or above 2.8 mg/l, providing sufficient oxygen for all cells. Oxygen was 458 supplied to the biofilm by diffusion through the silicone membrane, which also acts as a 459 support for the biofilm. As the biofilm grows it increasingly acts to resist the mass transfer of 460 nutrients to the inner regions of the biofilm. Calculations of oxygen penetration depth suggest 461 that the biofilm was sufficiently thick to prevent the penetration of oxygen to the region of the 462 biofilm located adjacent to the bulk liquid interface and support the possibility of oxygen 463 limitation.

464 At similar specific fluoroacetate loading rates the specific utilization rate of 465 fluoroacetate was decreased for biofilm cells in comparison to planktonic cells. A possible 466 explanation for this result is the decreased energy available to biofilm cells due to decreased 467 glycolate utilization, thus there is less energy produced for, among other requirements, the 468 production of fluoroacetate dehalogenase, and for the production of any permease necessary 469 for the transport of fluoroacetate into the cell. Therefore, a reduced glycolate utilization rate 470 due to oxygen limitation could ultimately affect fluoroacetate utilization. Decreased energy 471 availability for the transport of fluoroacetate into the cell during anaerobic growth may also 472 offer an explanation as to why only 1.4 mM of fluoroacetate was degraded under these 473 conditions when 8.5 mM was degraded in the same time period under aerobic conditions.

474 Cell cycle distributions show that there was no statistical difference in the percentage of cells in the B phase of growth at specific glycolate utilization rates between 4 and  $8 \times 10^{-12}$ 475 476 mM/CFU h for both biofilm and planktonic cells suggesting similar growth rates (Fig 7). If 477 biofilm growth rates of biofilm cells were decreased in comparison to planktonic cells at 478 similar specific glycolate utilization rates, then an increase in the number of cells occupying 479 the B phase of growth would be expected. This was not the case and the cells can be 480 considered to be in a similar physiological state. While some biofilm populations had 481 increased percentages of cells in the B phase of the cell cycle, these increases were probably due to carbon limitation as a result of the low carbon loading rates, between 0.06 and  $2 \times 10^{-12}$ 482 483 mM/CFU h, in these sections and not as a result of any intrinsic differences between the two 484 modes of growth. The planktonic system was not operated at these low carbon loading rates, 485 and consequently it was not possible to make comparisons between the two modes of growth 486 at these low carbon loading rates.

487 In conclusion the TBR was found to be a versatile system for determining the 488 performance of biofilm cells. The TBR allows the determination of local metabolite 489 concentrations and other parameters such as dry weight, CFUs, and biofilm thickness which 490 can then be use to assess biofilm performance. While other commonly used biofilm reactor 491 systems such as the rotating disk reactor, the capillary biofilm reactor, and the drip flow 492 biofilm reactor allow the determination of some of these parameters, none allow the 493 determination of so many parameters simultaneously. The identification of the overflow 494 metabolism in biofilm systems could have important implications in the treatment of 495 wastewater streams containing fluorinated compounds. In this case the overflow product was 496 glycolate. Glycolate subsequently acts as the carbon source and did not have any detrimental 497 effect on performance. However, the degradation of other fluorinated compounds, such as 498 fluoroaromatics, might result in the accumulation of toxic fluorometabolites, e.g.

499 fluorocatechol, which could detrimentally affect the performance of biofilm cells. Planktonic 500 cells were found to be superior to biofilm cells at degrading the xenobiotic with higher 501 fluoroacetate and glycolate utilization rates per CFU recorded at similar specific fluoroacetate 502 loading rates. This difference in performance can be explained by oxygen limitation in the 503 biofilm; however the high free fluoride concentrations recorded in the TBR may also have a 504 negative impact on performance. These results show that while planktonic cells were more 505 efficient at utilizing both fluoroacetate and the intermediate metabolite glycolate, the 506 advantages of biofilm systems for the degradation of xenobiotics, such as the enrichment of 507 slow growing species, may outweigh the superior performance of planktonic cells observed 508 here.

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  816
- 617
- 618 Figure Captions

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- 620 Figure 1: Schematic of Tubular Biofilm Reactor (TBR). Each section consists of a 40 cm of
- 621 silicone tubing with a total reactor length of 160 m.

623 Figure 2: The utilization of fluoroacetate involves the enzymatic cleavage of the carbon-624 fluorine bond with the production of glycolate. The specific utilization rate of glycolate q g is a combination of q an where carbon is incorporated into cell (anabolism), q en where carbon is 625 626 used to provide energy for growth, q m where carbon is used to provide energy for the 627 maintenance of cellular function not associated with growth (maintenance of intracellular 628 osmotic potential) but excludes any glycolate that is produced but not utilized (O m) and 629 represents the total carbon utilized. The specific fluoroacetate utilization rate includes q an, q e, 630 q<sub>m</sub> and O<sub>m</sub> and represents the total fluoroacetate degraded.

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632 **Figure 3:** Fluoroacetate defluorination via haloacetate dehalogenase

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Figure 4: Typical histogram of fluorescent intensity for planktonic cells, Multicycle software
was used to determine the B, C and D phases of the cell cycle. B phase cells have a single
copy of DNA, C phase cells are synthesising DNA and D phase cells have a double copy of
DNA and are predivision.

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**Figure 5:** Specific fluoroacetate (•) and glycolate (0) utilization rates for planktonic and specific fluoroacetate (•) and glycolate (□) utilization rates for biofilm cells at varying specific fluoroacetate loading rates. Regression analysis shows the superior performance of planktonic cells (slope=0.87, R<sup>2</sup>=0.90) in comparison to biofilm cells (slope=0.57, R<sup>2</sup>=0.91) at specific fluoroacetate loading rates between 2 and  $14 \times 10^{-12}$  mM/CFU h. 95 % confidence intervals (---) are shown.

645

646 **Figure 6:** Fluorescent images of biofilm stained with the respiratory indicator CTC (red) and 647 the DNA binding dye DAPI (A) and the corresponding depth profiles of the location of respiratory activity and biomass within the biofilm (B) show that respiratory activitydecreases towards the biofilm/bulk liquid interface.

Figure 7: Cell cycle distributions for (A) planktonic and (B) biofilm cells at a number of specific glycolate utilization rates. (□) B phase, (■) C phase and (■) D phase. Standard deviations were determined from a minimum of three independent biological samples. Planktonic specific glycolate utilization rates between 4-8 and 8-12 mM/CFU h correspond to growth rates of between 0.1-0.12 and 0.14-0.18 h<sup>-1</sup> respectively.

672 **Table 1.** Biofilm thickness, dry weight, CFUs, and density data after approximately 300 h of 673 growth on varying carbon loading rates. Carbon loading rates are used here and not 674 fluoroacetate loading rates to take it to account that some sections of the reactors have both 675 fluoroacetate and glycolate in the inlet feed.

6	Carbon	Initial reactor	Reactor	Thickness	Dry	CFU/ 40 cm	Density
	loading rate	fluoroacetate	section	(µm)	weight	section	(g/l)
	(mM/h)	concentration			(g/ 40 cm		
		(mM)			section)		
	0.000	10	4	12	0.0000	$1.12 \times 10^{9}$	0.00
	0.000	10	3	22	0.0016	$6.48 \times 10^9$	18.5
	0.000	20	4	29	0.0120	$6.20 \times 10^{9}$	108
	0.007	20	4	32	0.0128	$1.68 \times 10^9$	80.0
	0.017	10	2	150	0.0952	$4.40\times10^{10}$	169
	0.036	20	3	92	0.0624	$1.91\times10^{10}$	181
	0.036	20	3	85	0.0496	$1.52\times10^{10}$	154
	0.080	20	2	95	0.0728	$8.81  imes 10^9$	202
	0.081	20	2	136	0.0656	$2.72\times10^{10}$	128
	0.107	10	1	281	0.0696	$4.56\times10^{10}$	65.0
	0.168	20	1	120	0.0720	$1.92\times10^{10}$	159
	0.187	20	1	120	0.0872	$1.36\times10^{10}$	192
	0.304	50	4	134	0.0552	$6.16 \times 10^{9}$	108
	0.362	50	3	147	0.0768	$4.40 \times 10^{9}$	138
	0.412	50	2	96	0.0728	$1.12\times10^{10}$	201
	0.509	50	1	110	0.0648	$1.92\times10^{10}$	156









Figure: 4







