Factors influencing 4-fluorobenzoate degradation in biofilm cultures of *Pseudomonas knackmussii* B-13

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Abstract

Membrane aerated biofilm reactors (MABRs) have potential in wastewater treatment as they permit simultaneous COD minimisation, nitrification and denitrification. Here we report on the application of the MABR to the removal of fluorinated xenobiotics from wastewater, employing a *Pseudomonas knackmussii* monoculture to degrade the model compound 4-fluorobenzoate. Growth of biofilm in the MABR using the fluorinated compound as the sole carbon source occurred in two distinct phases, with early rapid growth (up to 0.007 h^{-1}) followed by ten-fold slower growth after 200 h operation. Furthermore, the specific 4-fluorobenzoate degradation rate decreased from 1.2 g $g^{-1}h^{-1}$ to 0.2 g $g^{-1}h^{-1}$, indicating a diminishing effectiveness of the biofilm as thickness increased. In planktonic cultures stoichiometric conversion of substrate to the fluoride ion was observed, however in the MABR, approximately 85 % of the fluorine added was recovered as fluoride, suggesting accumulation of 'fluorine' in the biofilm might account for the decreasing efficiency. This was investigated by culturing the bacterium in a tubular biofilm reactor (TBR), revealing that there was significant fluoride accumulation within the biofilm (0.25 M), which might be responsible for inhibition of 4-fluorobenzoate degradation. This contention was supported by the observation of the inhibition of biofilm accumulation on glass coverslips in the presence of 40 mM fluoride. These experiments highlight the importance of fluoride ion accumulation on biofilm performance when applied to organofluorine remediation.

Keywords

biofilm, fluorobenzoate, fluoride, membrane, biodegradation

1 Introduction

The membrane aerated biofilm reactor (MABR), in which oxygen is supplied to the biofilm solely from a gas permeable substratum, shows significant potential as a technology for high-rate biological wastewater treatment. In the MABR, the biofilm is naturally immobilized on an oxygen permeable membrane. Oxygen diffuses through the membrane into the biofilm where oxidation of pollutants, supplied at the biofilm-liquid interface takes place. The oxygen supply rate can be controlled by the intra-membrane oxygen partial pressure and membrane surface area. Several investigators have reported performance advantages of MABRs for simultaneous COD oxidation, nitrification, and denitrification (Hibiya et al. 2003; Semmens et al. 2003; Timberlake et al. 1988; Yamagiwa et al. 1994), high oxygen utilisation efficiencies (Pankhania et al. 1994) and high specific organic reaction rates (Brindle et al. 1999; Ohandja and Stuckey 2006). Much of the recent research has focused on nitrogen removal (Downing and Nerenberg 2008; Lackner et al. 2008), however there is also interest in the use of the MABR for the aerobic treatment of xenobiotics. MABRs are viewed as particularly favourable in this context because bubbleless operation minimizes the air-stripping of compounds with high Henry's law constants such as xylene (Debus and Wanner 1992) or acetonitrile (Li et al. 2008). The MABR is also of interest because the creation of and ease of manipulation of a defined oxic/anoxic micro-environment can be advantageous for the degradation of compounds with problematic intermediates such as perchloroethylene (Ohandja and Stuckey 2006).

In recent years the presence of fluorinated organic compounds in the environment has drastically increased as a result of the significant rise in the production of wide range of fluorinated pharmaceuticals and agrochemicals developed due to the unique and desirable properties of fluorine (Hansen et al. 2001; Muller et al. 2007). Environmentally-unfriendly incineration is the mainstay for the management of most fluorinated waste (dos Santos et al.

2001), thus alternative methods are required to treat post-production water contaminated with fluorinated compounds, and biological processes might be the most economically and ecologically sound option. Biofilm reactors are ideally suited to the biological treatment of xenobiotics. It has been shown that biofilms have a higher resistance to toxic compounds (Morton et al. 1998) and this attribute could be highly beneficial when treating fluorinated aromatic compounds as transformation intermediates are often toxic. Strains have been isolated from industrial environments that have degradation capabilities towards fluorinated compounds (Carvalho et al. 2005). One such species, isolated from a sewage treatment plant and shown to have the ability to degrade fluorinated aromatic compounds, is *Pseudomonas knackmussii*, also known as *Pseudomonas sp*. B13. The strain has been also reported to form biofilm (Nielsen et al. 2000) and with its degradation properties (Schreiber et al. 1980) is potentially an ideal microorganism for the removal of fluorinated aromatic compounds in wastewater treatment.

There are very few studies that have investigated the degradation of fluorinated compounds in biofilms, and none has investigated the degradation of fluorinated aromatic compounds in MABR system. Given the environmental significance of such compounds, we describe here investigations undertaken to evaluate the possible application of bacterial biofilms in the bioremediation of organofluorine-contaminated waste streams.

2 Materials and Methods

2.1 Medium and culture conditions

Pseudomonas knackmussii or *Pseudomonas* sp. B13 (DSM 6978) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Brunners mineral medium (www.dsmz.de, medium 457) supplemented with the appropriate carbon source (10 mM) was the growth medium except for where stated otherwise.

92 *2.2 Planktonic studies*

93 The planktonic growth rates were determined in 250 mL conical flasks with a working
94 volume of 50 mL and incubated at 30°C with shaking at 150 rpm. The flasks were inoculated
95 with 1 mL of a 24 h-old culture adjusted, only if needed, to an optical density of 0.8 at 660
96 nm with PBS (phosphate buffered saline). All planktonic trials were performed in triplicate.
97 Biomass was established by dry cell weight and colony forming units (CFU) measurements.

98 *2.3 MABR*

99 The MABR experiments were set-up as described previously by Heffernan et al. (2009b) with
100 a modification of the sampling port (SI, Fig 4). The system operation was commenced by
101 filling the reactor with 200 mL of medium and sterilisation of reactor by autoclaving at 121ºC
102 for 15 min in an Astel autoclave. Medium for the continuous phase of operation was sterilised
103 separately by autoclaving at 121ºC for 15 min in a 10 L carboy, which was subsequently
104 connected aseptically to the reactor vessel. To prepare the inoculum; cells were first grown
105 for 24 h in batch culture prior to reactor inoculation to obtain the exponential phase of
106 growth; 10 mL of this culture was adjusted to a turbidity of approximately 0.8 at 660 nm in
107 PBS and used to inoculate the reactor. Following inoculation the system was operated in
108 batch mode for 48 h after which time the flow of medium was initiated at a flow rate of 40
109 mL h^{-1} and maintained throughout the biofilm accumulation. Visible biofilm appeared within
110 48 h from commencing the feed. Experiments involving a change of the flow rate and
111 pressure were performed after the biofilm had reached a steady state, which was established
112 by thickness measurements. The reactor effluent was sampled with a 5 mL luer lock syringe
113 attached to the circulation loop and analysed for pH, optical density (OD) and dissolved
114 oxygen concentration (DO). The culture supernatant was also obtained by centrifuging the
115 remaining liquid sample and stored at -20ºC until analysis of the fluoride ion and other
116 fluorometabolites were conducted.

2.4 *Tubular Biofilm Reactor (TBR)*

The TBR experiments were set-up with some modifications of the system described previously by Heffernan et al. (2009a). Silicone tubing was sourced from VWR and Altec. The system was equipped only with one sample port located at the downstream section of the 90 cm reactor tubing and an additional 300 mL Duran bottle was introduced as an inoculation tank containing 200 mL of medium (SI, Fig 5), which was sterilised and then inoculated with 5 mL of 24 h-old cells (OD = 0.8). Freshly inoculated medium was pumped through the system and after 48 h the feed was switched on and the first sample of culture supernatant was taken. TBR experiments with 10 mM of 4-fluorobenzoate as the carbon source were conducted for 216, 288, 360, 408, 432, and 504 h. Biofilm appeared within 96 h from the reactor inoculation. Samples were collected every 24 h during continuous culture using a luer lock syringe connected to the sampling port. DO, OD and pH were measured and the remaining liquid sample was centrifuged and the supernatant stored frozen until analysis of the fluoride ion and other fluorometabolites were conducted.

The initial investigation of free fluoride accumulation in TBR grown biofilm was performed in two experiments using medium containing 10 mM 4-fluorobenzoate as a carbon source. After 216 h operation of these two replicates was terminated and content of each reactor was harvested as three fractions: planktonic culture suspended in media (F1), sloughed off biofilm (F2) and mature biofilm (F3). F1 was obtained by draining the reactor tubing; F2 by washing cell content out with known volume of deionised water and F3 by pinching biofilm out and washing it out with known volume of deionised water. After each fraction was centrifuged, supernatants were examined for free fluoride ion concentration. Precipitated cells from each fraction were sonicated for 3 min (Ika Labortechnik U200S control) and centrifuged. Fluoride ion was measured in cell free extracts. Organic

fluorometabolites were extracted with ethyl acetate from cell extracts and detected by ^{19}F NMR.

2.5 Biofilm thickness measurement

Biofilm thickness measurements in the MABR system were performed using the method described previously by Syron and Casey (2008b). The value obtained from the raw thickness was linearized (ζ) by using the equation $\zeta=(r_o+\delta)\ln((r_o+\delta)(r_o)^{-1})$, where δ is the recorded thickness and r_o is the radius of the silicone tube; the biofilm accumulation rate was then calculated from this value.

Biofilm thickness in the TBR was established after each experiment was terminated and in a different manner than the online biofilm thickness measurement in the MABR system. Thickness analysis was performed using Able Image Analyser software (Mu Labs, Slovenia) on biofilm light microscopy images taken with an Olympus DP70 digital camera (4x magnification) by measuring the distance from the membrane to the biofilm liquid interface. The microscopy biofilm images were obtained after the reactor tubing (together with biofilm grown inside it) was cut and cryoembeded (embedded in freezing medium and frozen), the silicone tubing was removed and biofilm sample was sliced. The procedure was previously described by Heffernan et al. (2009a). From each section of the reactor six phase contrast images were analysed for thickness with 45 measurements taken from each image. These measurements were then averaged to give a final thickness.

2.6 *Biofilm cultivation in six well plates*

Glass cover slips were treated with UV light, place in individual wells of 6-well plates and immersed in 8 mL sterile medium containing either 4-fluorobenzote (10 mM), benzoate (10 mM) or benzoate (10 mM) plus sodium fluoride (40 mM). Subsequently, the medium was inoculated with 1 mL of 24 h-old culture (OD = 0.8 at 660 nm) and incubated on a rocking

platform at 30°C. Control wells contained medium that was not inoculated. At each sampling time (10, 24, 34, 48, 58 and 72 h) cover slips were removed and washed with phosphate buffered saline (PBS). The cover slips were stained with crystal violet and DAPI (4',6-diamidino-2-phenylindole) and microscopically examined using an Olympus BX51 epifluorescence microscope. The area of biofilm coverage of each slide was calculated by dividing the sum of all unit areas by the total area of a slide (141,050 μm^2). The average biofilm coverage represents the average value obtained from 6 images, where each image represents separate slide taken from a well of a 6 well plate at each sampling time.

2.7 *Substrate and product analysis*

Free fluoride concentration was measured by Fluoride/Fluoride Combination Electrode (Orion model 94-09) following the method described by Cooke (1972). The electrode was calibrated using NaF standards (1 mM, 10 mM and, if needed, 100 mM) in a mixture of H_2SO_4 (1 M) and KNO_3/trisodium citrate buffer (0.5 M). Sample preparation involved mixing 1 mL of supernatant (or cell extract) with 1 mL of H_2SO_4 (1 M) solution and 8 mL of a KNO_3/trisodium citrate buffer (0.5 M).

4-Fluorobenzoate and benzoate concentrations were determined by High Pressure Liquid Chromatography (HPLC) using a Varian ProStar system. Supernatant (10 μm) was eluted from a reverse phase column C18 (4.6 x 150 mm, 5 μm column Thermo Hypersil) using phosphoric acid (1g L^{-1}) and acetonitrile (60:40) as the eluent. The 254 nm wavelength was monitored. The retention time under these conditions was 3.60 minutes for 4-fluorobenzoate and 3.29 minutes for benzoate. 4-Fluorobenzoate was obtained from Fluorochem (Derbyshire, UK) other chemicals and various media components were obtained from a number of sources including BDH, Oxoid and Sigma-Aldrich.

189 Culture supernatants and extracts thereof were analysed by ^{19}F nuclear magnetic
190 resonance spectroscopy (^{19}F NMR), using D_2O as a solvent. Resonances detecting free
191 fluoride, 4-fluorobenzoate and 4-fluorocyclohexadiene-*cis,cis*-1,2-diol-1-carboxylate (4FDC)
192 appear at δ -120, -110 and -116 ppm, respectively (Boersma et al. 2004; SI, Fig 6). All ^{19}F
193 NMR analyses were performed using a Varian 400 MHz spectrometer.
194
195 **3 Results**

196 *3.1 Development of biofilm in MABR*

197 *P. knackmussii* was adapted for growth on 4-fluorobenzoate by continuous subculturing in
198 medium containing 10 mM of the fluorinated substrate, eventually reaching a growth rate of
199 0.22 h^{-1}. This adapted strain was employed in all planktonic studies and in the establishment
200 of the biofilm cultures.

201 MABR experiments were conducted for 264 h (reactor I) and 600 h (reactors II and
202 III), where 10 mM 4-fluorobenzoate was the sole source of carbon. Growth of the biofilm
203 was assessed by measuring the thickness, and in the reactors II and III two distinct growth
204 phases were observed: a period of relatively fast growth up to 200 h (0.004 and 0.007 h^{-1},
205 respectively) followed by a slower (factor 10x) growth phase (Fig 1A). Effluent from the
206 reactors was collected every 24 hours and the concentrations of fluoride ion and 4-
207 fluorobenzoate were measured, and used to calculate a specific utilisation rate (Fig 1B).
208 Although the biofilm thickness increased throughout the period of operation, the
209 concentration of 4-fluorobenzoate (Fig 1C) and fluoride ion in the effluent stabilised after
210 approx. 100 h, thus the specific degradation rate decreased from 1.2 g $g^{-1}h^{-1}$ in the early stage
211 of biofilm growth (<200 h) to 0.2 g $g^{-1}h^{-1}$ as the biofilm matured. Furthermore it was
212 established during operation of reactor III, that there was no correlation between substrate
213 degradation and oxygen supply; the highest specific utilisation rate was established at a

pressure of 1 bar pure oxygen but only during the initial biofilm growth. The first increase of oxygen pressure from 0.4 to 0.7 bar resulted in increase of DO found in spent media from 3 to 6 mg/L. After the oxygen pressure was again increased the DO decreased back to the previous level of 2-3 mg/L and did not change overall during the rest of experiment even when the oxygen pressure was regularly elevated and declined. Therefore, some other factor(s), such as the accumulation of a toxic compound within the biofilm, was the cause of the poor substrate degradation rate (Adebusoye et al. 2008; Baggi and Zangrossi 1999; Miguez et al. 1995). Approximately 85 % of the fluorine initially added could be accounted for in terms of the fluoride ion and 4-fluorobenzoate; ^{19}F NMR analysis of the effluent revealed only these metabolites (not shown), thus it is possible that a fluorinated intermediate, or fluoride ion, might accumulate in the biofilm. To investigate this, a TBR was employed because it facilitates recovery of biofilm for analysis of metabolites.

3.2 Characteristics of 4-fluorobenzoate degradation in TBR

P. knackmussii was grown in the TBR with 10 mM 4-fluorobenzoate as a source of carbon for experiments of 216-504 h duration and terminated when the system reached steady state based on unvarying and low OD values of the effluent. Initial attachment, microcolony formation, development of mature biofilm structure, sloughing events and biofilm recovery were observed to take place during each TBR experiment. Since each experiment was terminated at a different time it was possible to observe a relationship between cultivation time and biofilm thickness (Fig 2). The utilization of 4-fluorobenzoate was monitored by free fluoride and 4-fluorobenzoate measurements in the effluent, which together with biofilm dry cell weight measurements were used to calculate final specific utilisation rates.

The specific utilization decreased with biofilm increasing thickness, similar to what was observed in the MABR. A doubling of the thickness, from 25-50μm, resulted in a 4-fold decrease in the degradation rate (Fig 2). This difference is unlikely to be explained by

239 substrate or oxygen limitation given the low thickness of the TBR grown biofilms.
240 Furthermore the total fluorine (fluoride ion plus 4-fluorobenzoate) recovered in culture
241 supernatants was approximately 85 % of the starting substrate concentration. ^{19}F NMR
242 analyses of the effluent demonstrated that these comprised the major fluorine components,
243 although a very small signal at δ -116 ppm (4-fluorocyclohexadiene-*cis,cis*-1,2-diol-1-
244 carboxylate) was also observed (SI, Fig 6).

245 Fluoride ion accumulation has been observed in oral biofilms (Engstrom et al. 2002;
246 Watson et al. 2005) and it is a known enzyme inhibitor (Marquis et al. 2003; Nordstrom et al.
247 2009; Phan et al. 2002), thus the concentration of fluoride ion was determined in the biofilm.
248 Three fractions of *Pseudomonas knackmussii* culture obtained from the two TBR experiments
249 operated for 216 h were analysed; the planktonic cells collected along with spent medium
250 (F1); the biofilm which detached after washing the TBR tubing with deionised water (F2);
251 and the biofilm that was scraped off the TBR tubing (F3). In total, 0.095 mmol total fluoride
252 ion was measured most of which was directly associated with the biofilm (Table 1). Fluoride
253 ion was also measured in the biofilm extracts from TBR experiments that were conducted for
254 216-504 h. The amount of free fluoride as well as its total concentrations retained in the
255 biofilm were found to increase with time, and consequently with thickness, from 0.14 to over
256 0.25 M (Fig 2). Mass balance calculated for those TBR experiments indicates that
257 accumulation of fluoride ion does not account for all of the fluorine in the biofilm (Table 2).
258 At least some of the fluorine is also present as 4-fluorobenzoate (SI, Fig 7), and the remainder
259 may be in the form of polymeric substances arising from auto-oxidation of fluorocatechols,
260 which are known intermediates of fluorobenzoate degradation, and are not typically
261 observable by ^{19}F NMR.

3.3 The effect of fluoride ion on planktonic growth rates and biofilm formation.

The effects of fluoride have been studied in a variety of microorganisms, but nothing is known about the effects of the ion in *P. knackmussii*, thus experiments were undertaken by supplementing the medium with sodium fluoride in planktonic batch growth trials and biofilm cultures. To compare and eliminate possible growth limitations occurring as a result of fluorinated substrate degradation, benzoate was used as an alternative sole source of carbon in some experiments. Growth and specific utilisation rates were calculated in planktonic cultures incubated with 10, 20 and 40 mM NaF, by measuring dry cell weight and CFU (Table 3). In all planktonic batch experiments all applied fluorine, as 4-fluorobenzoate, was recovered in the culture supernatant as free fluoride ion at the end of logarithmic phase. Growth rates decreased as the fluoride ion increased, and the differences in growth rates were much more marked when benzoate was the carbon source. Similarly the specific utilisation rates of the substrate decreased as the concentration of fluoride increased.

The effect of fluoride ion on biofilm development was investigated by culturing the bacterium in six-well plates containing a glass coverslip, with medium containing benzoate and supplemented with 40 mM sodium fluoride. This additional fluoride concentration was established to have a clear negative impact on *P. knackmussii* biofilm culture. Crystal violet staining followed by microscopy and image software analysis was used to calculate the proportion of area covered by biofilm in all 6-well plate experiments (Fig 3). The average percentage coverage area based on image analysis corresponded well to the observed visual result for each slide (SI Figs 1-3) and complemented the OD recorded for planktonic cells; as biofilm grew on the glass cover slide the amount of the biomass in suspension decreased. It is clear that in the presence of 40 mM fluoride ion there is a dramatic reduction in coverage area.

4 Discussion and conclusions

Organofluorine compounds are used in an extensive range of applications, and consequently are widespread in the environment (Key et al. 1997). Microorganisms have long been known to degrade organofluorine compounds (Murphy 2010) thus have considerable potential in bioremediation of polluted sites and waste streams. However, relatively few investigations have been conducted on the use of biofilms for organofluorine degradation (Emanuelsson et al. 2006; Osuna et al. 2008). The main objective of the present research was to assess the performance of continuously operated bench-scale biofilm reactors for biodegradation of fluorinated compounds. *Pseudomonas knackmussii* was used as a model organism since its utilisation abilities towards halogenated compounds is well studied (Dorn and Knackmuss 1978; Schmidt et al. 1980; Schreiber et al. 1980). The MABR was chosen as the main system for investigation, since high loading rates can be applied and high oxygen transfer efficiencies are achievable through bubbleless aeration. (Syron and Casey 2008a).

4.1 MABR performance

P. knackmussii planktonic and biofilm culture was shown to utilise 10 mM of 4-fluorobenzoate as a source of carbon. However, in the MABR the calculated specific 4-fluorobenzoate degradation rates unexpectedly declined with increasing biofilm thickness. A similar observation was made by Heffernan et al. (2009b) while investigating fluoroacetate degradation in *P. fluorescens* in MABR. Mathematical modelling suggested that the rate of fluoroacetate degradation was affected by oxygen limitation and fluoride ion accumulation, but in the present study increasing the intra-membrane oxygen pressure in the MABR did not affect the oxygen transfer rate. Furthermore, *P. knackmussii* biofilm cultivated in MABR on 4-fluorobenzoate did not achieve the thickness of *P. fluorescens* biofilm (1000 μm). Thus some other factor(s) causes the specific degradation rate to decline.

4.2 Free fluoride accumulation in biofilm continuous culture

There was a lower concentration of total fluorine (fluoride ion plus 4-fluorobenzoate) in the spent medium from the MABR experiments than in the starting medium, indicating that some 'fluorine' had accumulated in the biofilm. Thus a possible reason for declining degradation ability of biofilms in MABR was accumulation of free fluoride, which has been observed in dental plaques (Engstrom et al. 2002; Petersson et al. 2002; Twetman et al. 2003) but not previously reported in *P. knackmussii* biofilms. Since it is difficult to examine biofilm composition in the MABR system even after the experiment is terminated, biofilm grown in TBR was examined and high concentration of fluoride ion of up to 0.25 M were measured. Furthermore, accumulation of fluoride ion in the biofilm was determined to occur over time, and at such high concentrations might inhibit several key enzymes resulting in a diminished capability of the cells to degrade substrate.

4.3 Fluoride ion impact on planktonic and biofilm culture

Fluoride ion has been reported to impair transport mechanisms in bacteria (Marquis et al. 2003) and inhibit action of several enzymes (Belli et al. 1995; GuhaChowdhury et al. 1997; Phan et al. 2002; Todd and Hausinger 2000). Ochoa-Herrera et al. (2009) investigated the effect of fluoride on microorganisms associated with wastewater systems, including denitrifying bacteria, aerobic heterotrophs and methanogens, and found that the groups of organisms investigated varied in their sensitivity to the ion. Thus, it was necessary to verify the influence of free fluoride concentrations on the planktonic and biofilm growth of *P. knackmussii*. Fluoride concentrations of 20 mM inhibited the growth of the strain in planktonic culture, and the formation of biofilms on coverslips was severely affected by the presence of 40 mM fluoride, strongly suggesting that the fluoride that accumulated in the biofilm as a result of 4-fluorobenzoate degradation in the MABR and TBR systems was

responsible for the diminished degradation capacity, and highlights the problem of using such a system to remediate organofluorine-contaminated wastewater streams.

5. Conclusions

The biodegradation of 4-fluorobenzoate was investigated in planktonic and biofilm cultures using *Pseudomonas knackmussii*, a strain originally isolated from a wastewater treatment plant. It was established that the performance of the biofilm culture was comparable to planktonic culture. However, the specific utilisation of the substrate decreased with increasing biofilm thickness. The presence of fluoride ion, as the main product of 4-fluorobenzoate utilisation, was shown to be detrimental to planktonic growth and biofilm development. High concentrations (up to 0.25 M) of free fluoride were found to be retained within the biofilm and probably contributed to the decrease of the specific degradation rates. This work has broader implications for the use of biofilm-based wastewater treatment systems where organofluorine xenobiotics are to be degraded. Further investigation would be needed to establish the effect of fluoride accumulation on the degradation of organic matter in biofilm treatment processes for industrial wastewater.

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475 **Figure legends**

476 **Figure 1** Linearised biofilm thickness measurements (Fig A) reveal two distinct stages (A
477 and B) of biofilm development, which biomass accumulation is correlated with declining
478 specific utilisation rates (Fig B) calculated as grams of utilized 4-fluorobenzoate by grams of
479 dry cell weight per hour in MABRs I (—■—), II (····□····) and III (—□—) operated with
480 10 mM 4-fluorobenzoate as a source of carbon. Substrate concentration measured in spent
481 media supernatant is also shown (Fig C).

482 **Figure 2** Data illustrating free fluoride accumulation increasing in time and its potential
483 negative impact on utilization abilities of *P. knackmussii* biofilm cultivated on 10 mM 4-
484 fluorobenzoate in TBR systems operated for 216, 288, 360, 408, 432 and 504 h. Biofilm
485 thickness (□)obtained from image software analysis and free fluoride concentration
486 (■) measured in biofilm cell free extract were established after each experiment was
487 terminated. Final specific utilization rates (▫) were calculated for each TBR experiment
488 as a gram of substrate utilized by gram of dry cell weight per hour. Presented thickness
489 (——), fluoride ion concentration (·····) and final specific utilisation (—·—) regression
490 plots r^2=0.85, r^2=0.6 and r^2=0.9, respectively.

491 **Figure 3** Average percentage coverage area data collected for 6-well plate experiments where
492 media contained: 10 mM 4-fluorobenzoate (▭), 10 mM benzoate (▭) and 10 mM
493 benzoate as a sole source of carbon plus 40 mM sodium fluoride (▬), complemented
494 with OD (—■—) recorded for sample supernatants and investigated substrate concentrations

495

Table 1. Free fluoride amount measured in *Pseudomonas knackmussii* culture retrieved from TBR system operated for 216 h. Data shown as average of two replicate experiments.

Fraction	Volume (mL)	Fluoride ion amount (mmol)	
		Culture supernatant	Cell extract
F1	6.164	0.004	0.038
F2	0.019	0.007	0.018
F3	0.175	0.003	0.025
Sum	6.359	0.014	0.081

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Table 2. Fluoride mass balance for each individual TBR experiment.

TBR operation (h)	Amount of fluorine			
	As substrate (mmol)	In spent medium (mmol)	In biofilm (F3) (mmol)	Recovered (%)
216	13.5	11.4	0.025	85
312	27.9	23.1	0.020	83
360	35.1	27.9	0.040	80
408	42.3	34.5	0.036	82
504	56.2	46.4	0.054	83

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Table 3. Specific growth rates based on CFU counts and specific utilization rates calculated as gram of utilised substrate by gram of dry cell weight of biomass per hour obtained in growth trials, where planktonic culture of *P. knackmussii* was grown on 10 mM 4-fluorobenzoate and benzoate as sole sources of carbon with 0, 10, 20 and 40 mM supplemental sodium fluoride. All trials were performed in triplicates.

Sodium fluoride (mM)	μ_{max} (h^{-1})	q ($g\ g^{-1}h^{-1}$)	μ_{max} (h^{-1})	q ($g\ g^{-1}h^{-1}$)
	4-Fluorobenzoate		Benzoate	
0	0.26	2.37	0.60	2.21
10	0.22	1.49	0.30	0.92
20	0.21	1.38	0.29	0.81
40	0.18	1.34	0.17	0.43

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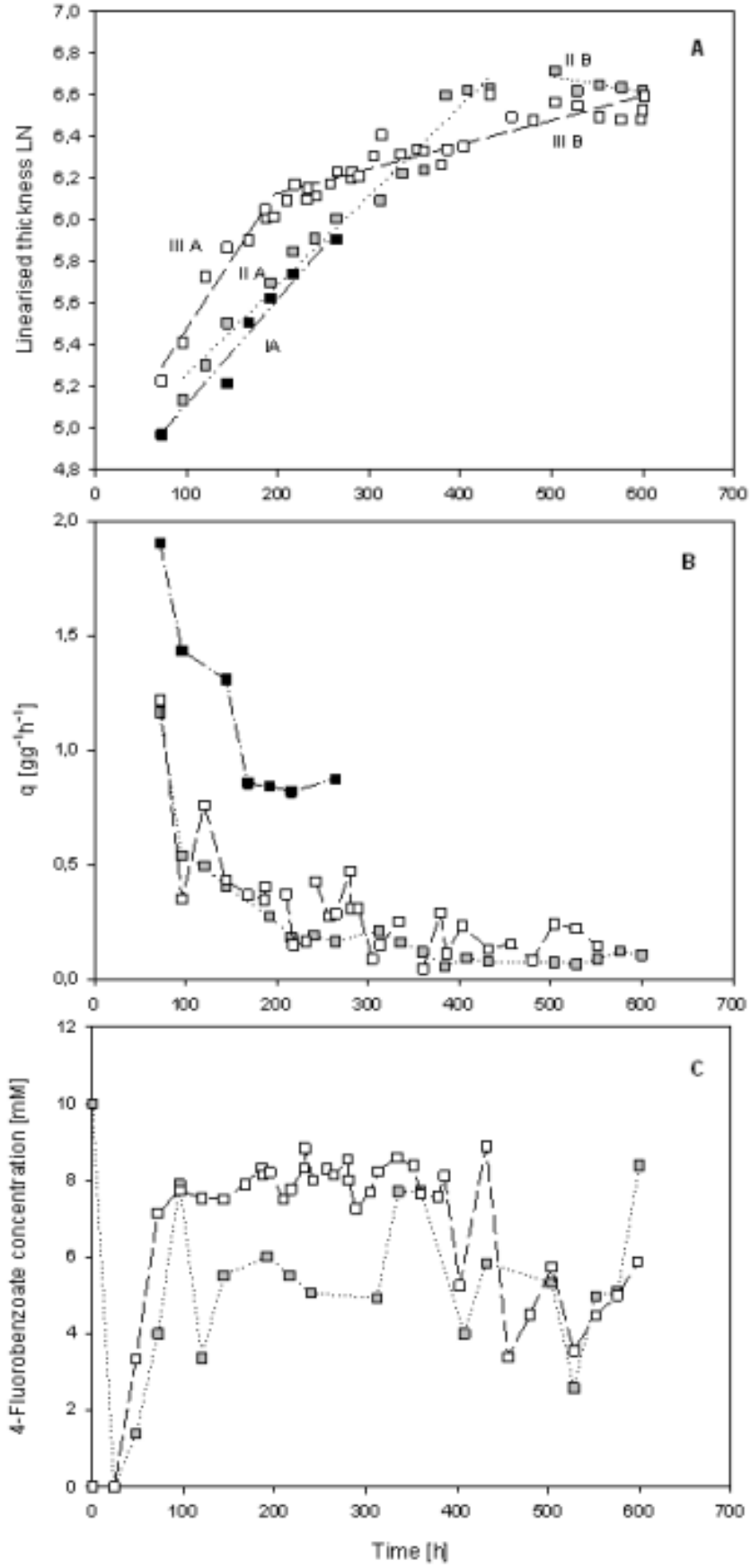
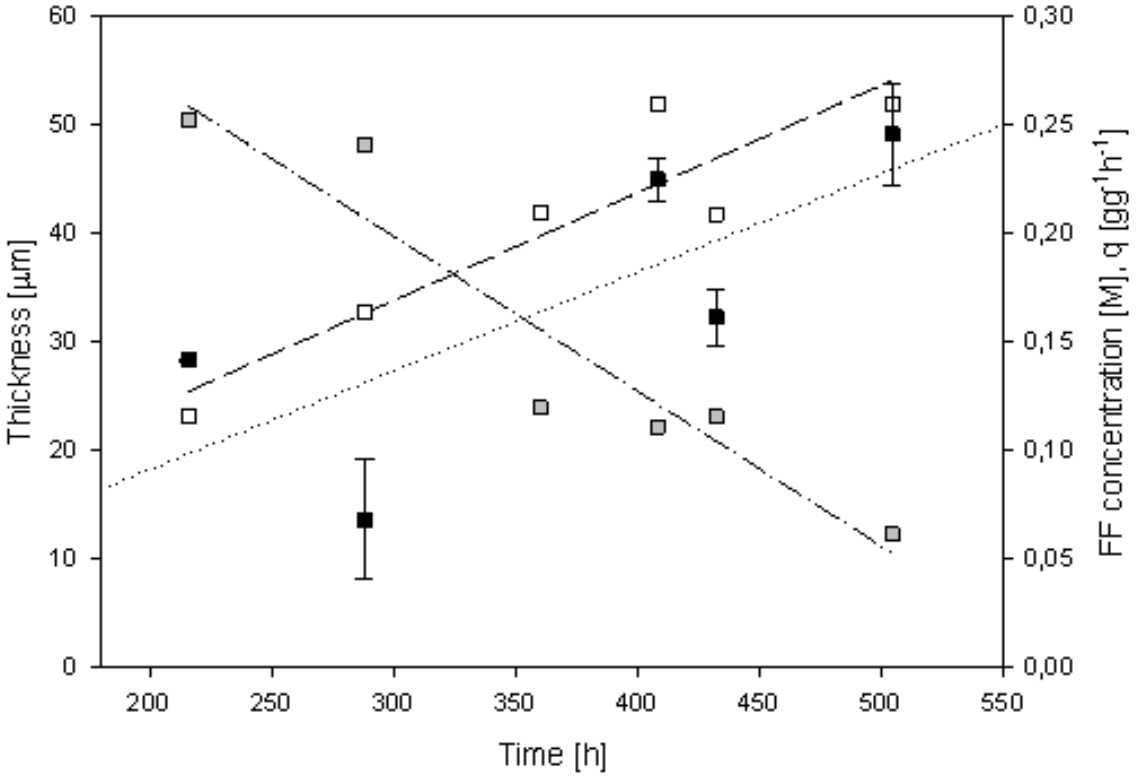

A
Linearised thickness LN
III A
II A
I A
II B
III B
B
q [gg⁻¹h⁻¹]
C
4-Fluorobenzoate concentration [mM]
Time [h]

504
505


Thickness [µm]
FF concentration [M], q [gg^{-1}h^{-1}]
Time [h]
0
10
20
30
40
50
60
0,00
0,05
0,10
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0,20
0,25
0,30
200
250
300
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400
450
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550

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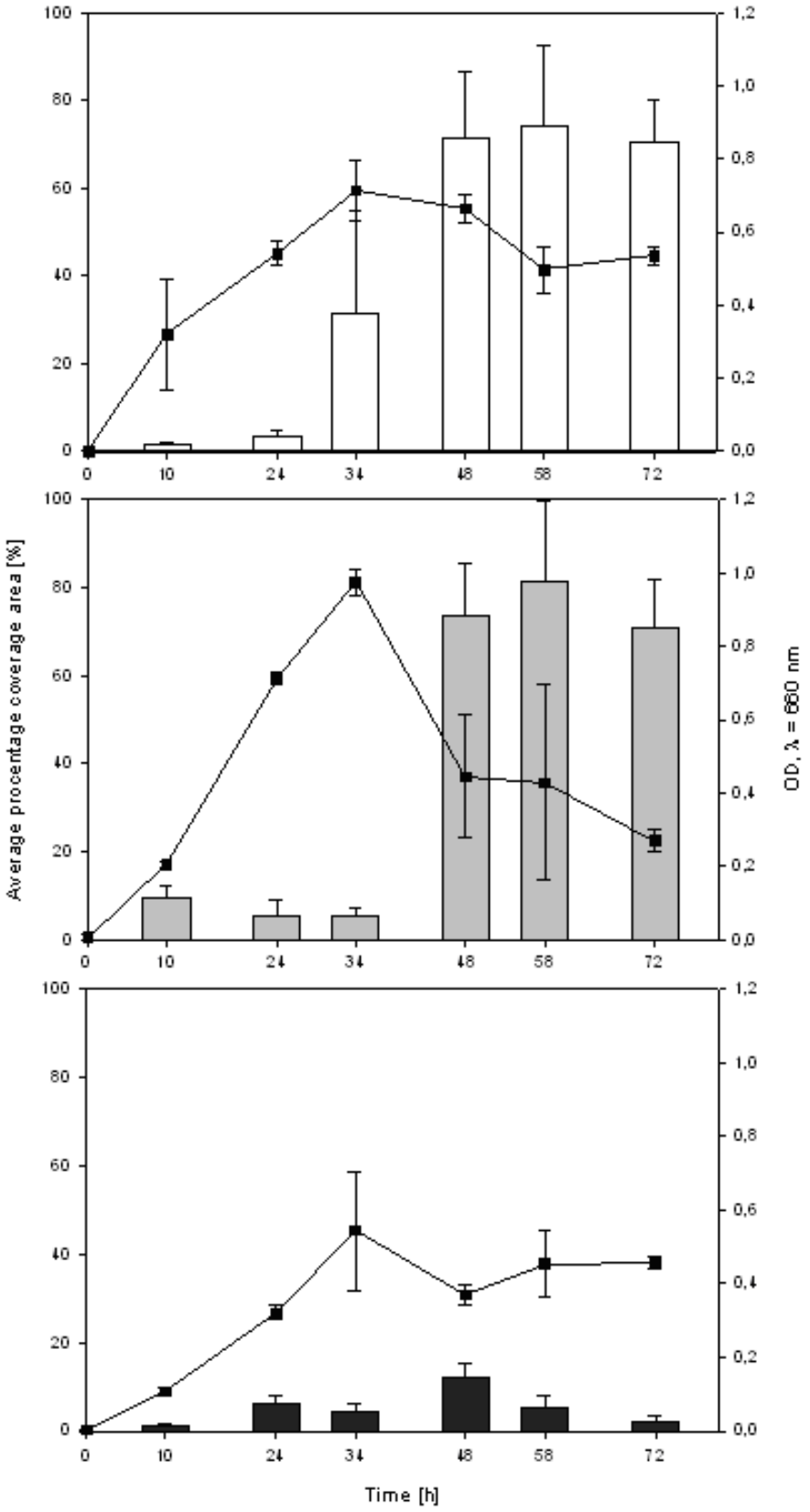
Average procentage coverage area [%]
OD, λ = 660 nm
Time [h]
0
10
24
34
48
58
72
0,0
0,2
0,4
0,6
0,8
1,0
1,2
20
40
60
80
100

508

SUPPLEMENTARY INFORMATION

Factors influencing 4-fluorobenzoate degradation in biofilm cultures of *Pseudomonas knackmussii* B-13

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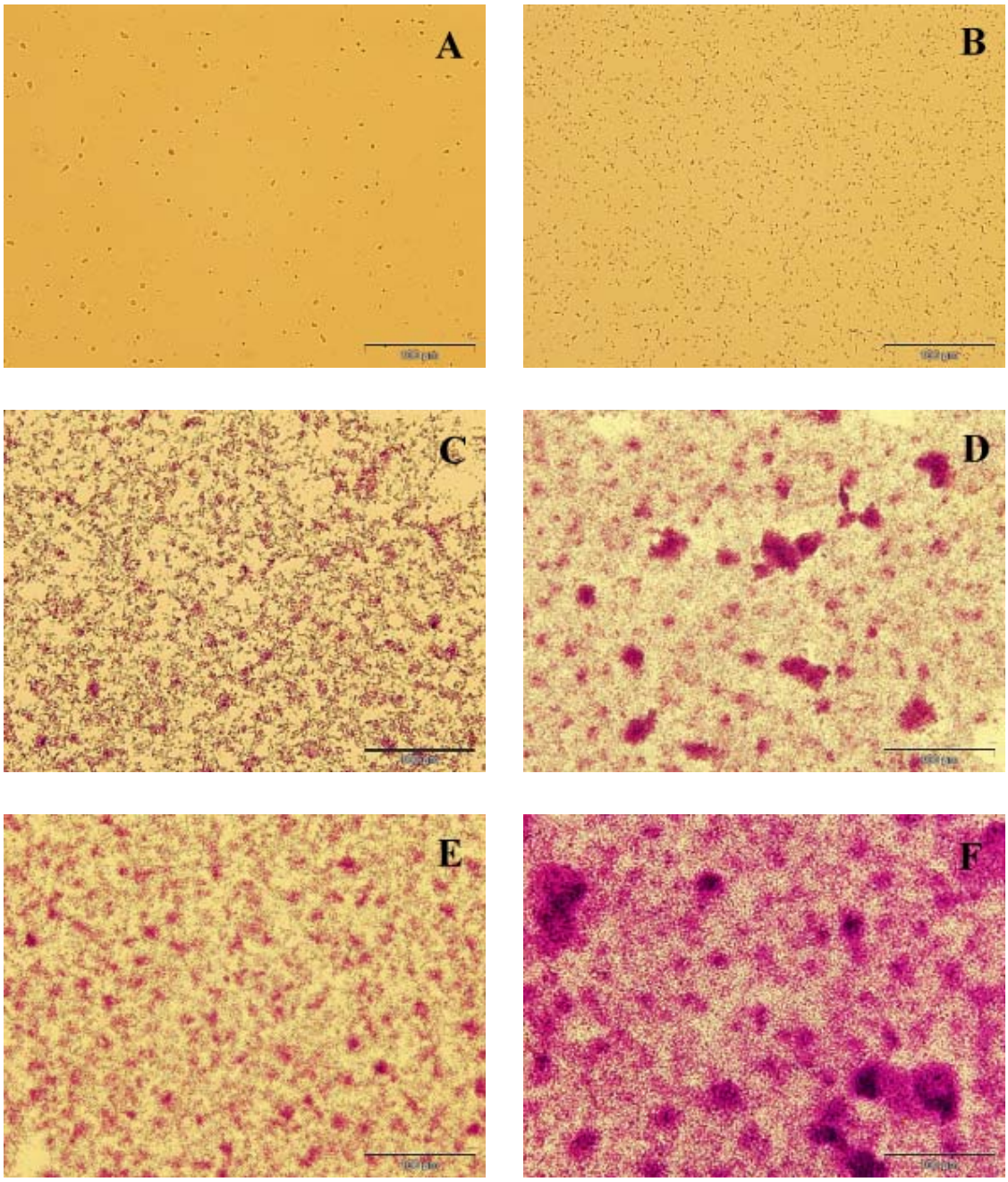
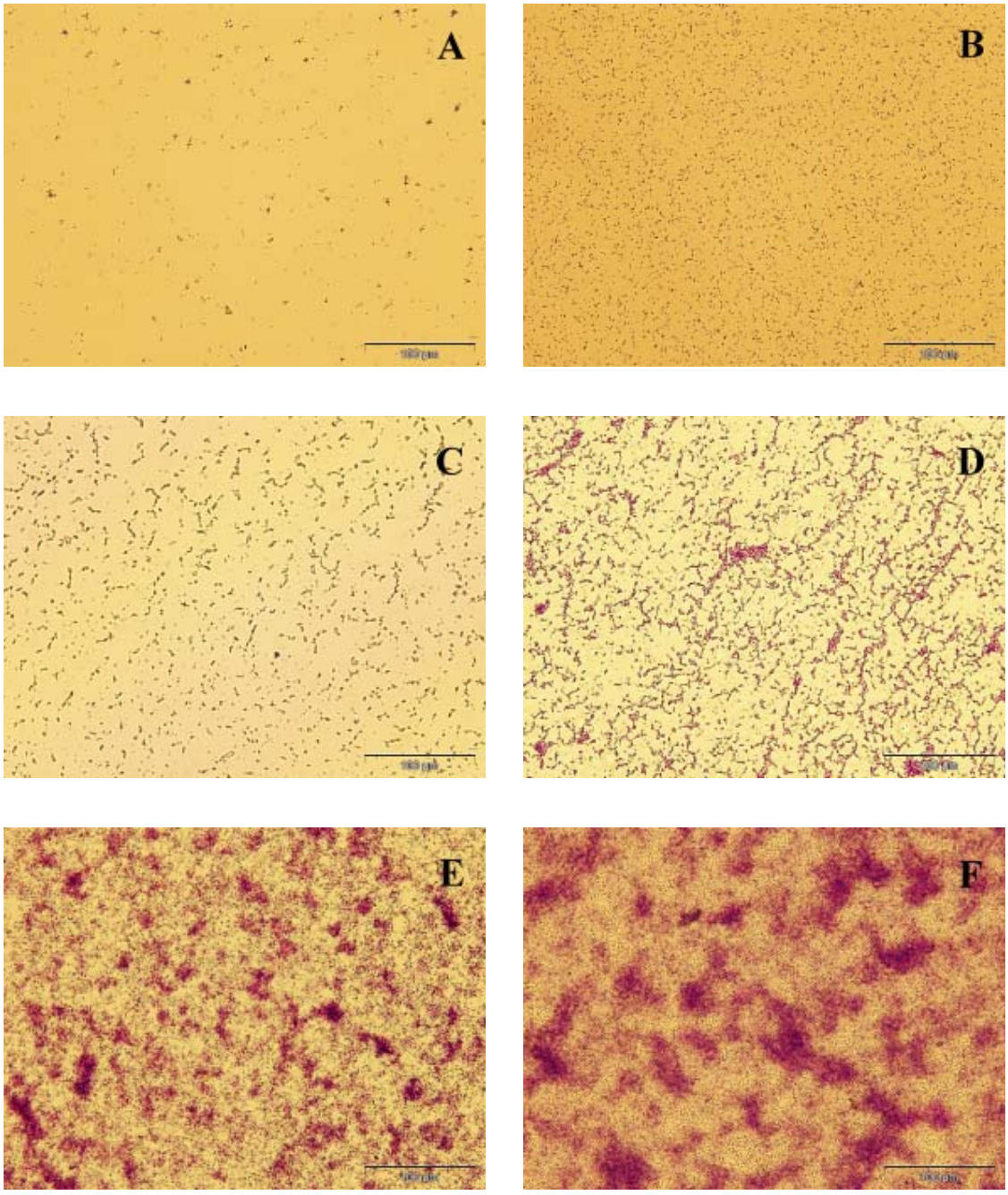
In both, 4-fluorobenzoate and benzoate 6-well plate experiments (Figures 1 and 2), where no addition of fluoride ion was used, biofilm development preceded in similar timeframe and each stage of its growth was recorded. Images taken after 10 h of culture incubation present the initial cell attachment as scattered singular cells on the cover slip surface were visible (Figures 1A and 2A). The number of singular cells covering the glass surface was higher in the next sample taken after 24 h (Figures 1B and 2B) and biofilm microcolonies appeared after 34 h (Figures 1C and 2C). Subsequently larger microcolonies started to merge to form a relatively uniform microbial layer after 48 h (Figures 1D and 2D) and fully grown biofilm was formed after 58 h (Figures 1E and 2E). Mature biofilm develops as a highly dynamic structure and it is typical to observe localised regions of high cell density within the biofilm, these are indicated by more intense staining visible on image taken for 72 h sample (Figures 1F and 2F).

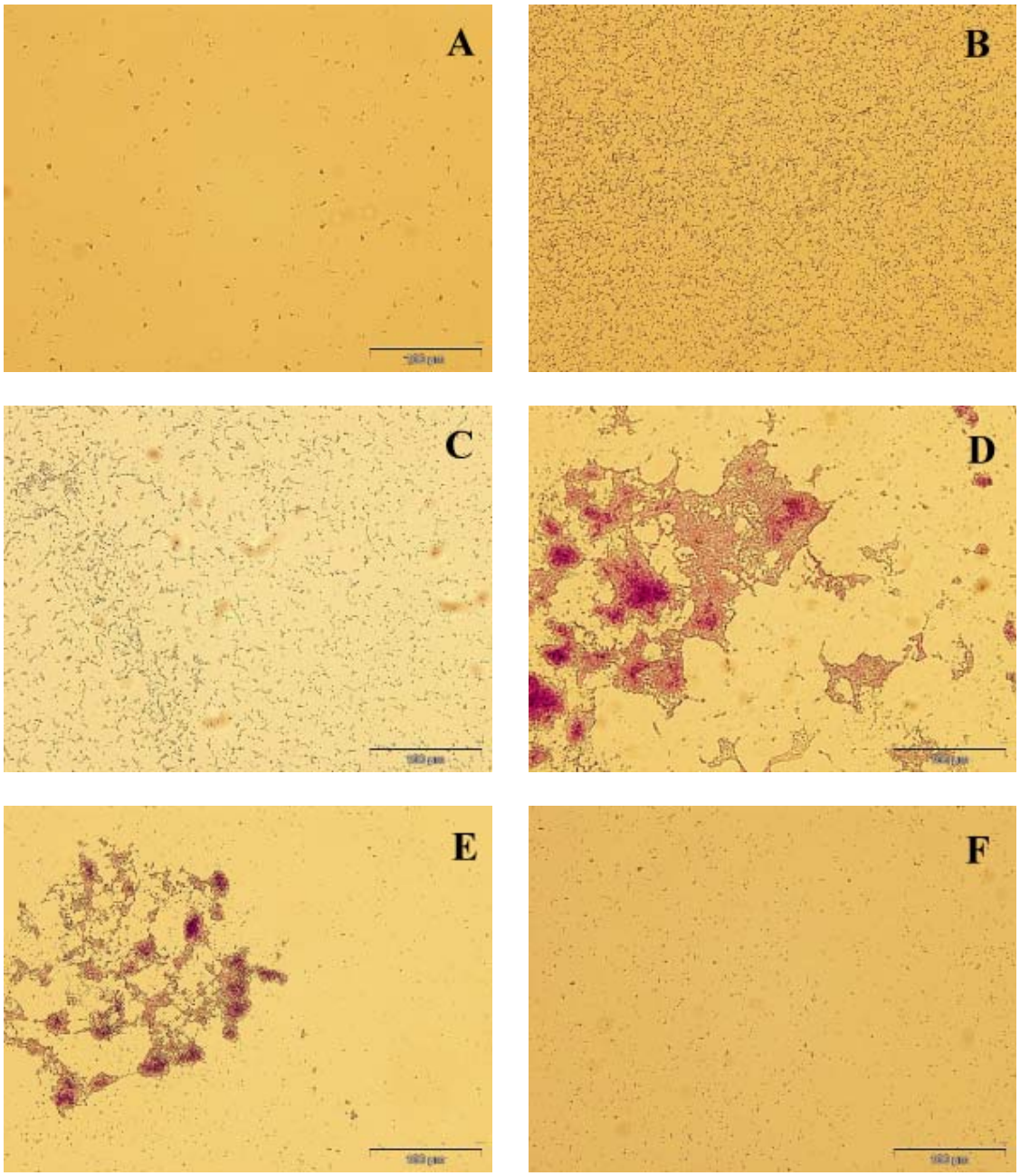
During the investigations of *P. knackmussii* biofilm formation in 6-well plates with 40 mM sodium fluoride addition and where 10 mM 4-benzoate was used as a sole source of carbon, the first three images (Figure 3A, B and C), corresponding to 10 h, 24 h and 34 h, respectively, show biofilm formation patterns (in terms of singular attachment and microcolonies) to be broadly similar to those observed for cultures grown on 4-fluorobenzoate or benzoate without any sodium fluoride supplements. Despite the fact that images recorded after 48 h showed a patchy biofilm structure, where microcolonies were well defined, singular attached cells were still noticeable (Figure 2D). Images obtained after 58 h and 72 h incubation demonstrate a significant change in biofilm formation pattern; biomass detachment and reorganization of the structure towards single cell attachment (Figures 3E and F). Thus percentage biofilm coverage increased but was four times lower than that observed in previous trials in which no supplemental fluoride was added. The decrease in percentage biofilm coverage can be attributed to detachment as confirmed by the increased OD in the suspension.



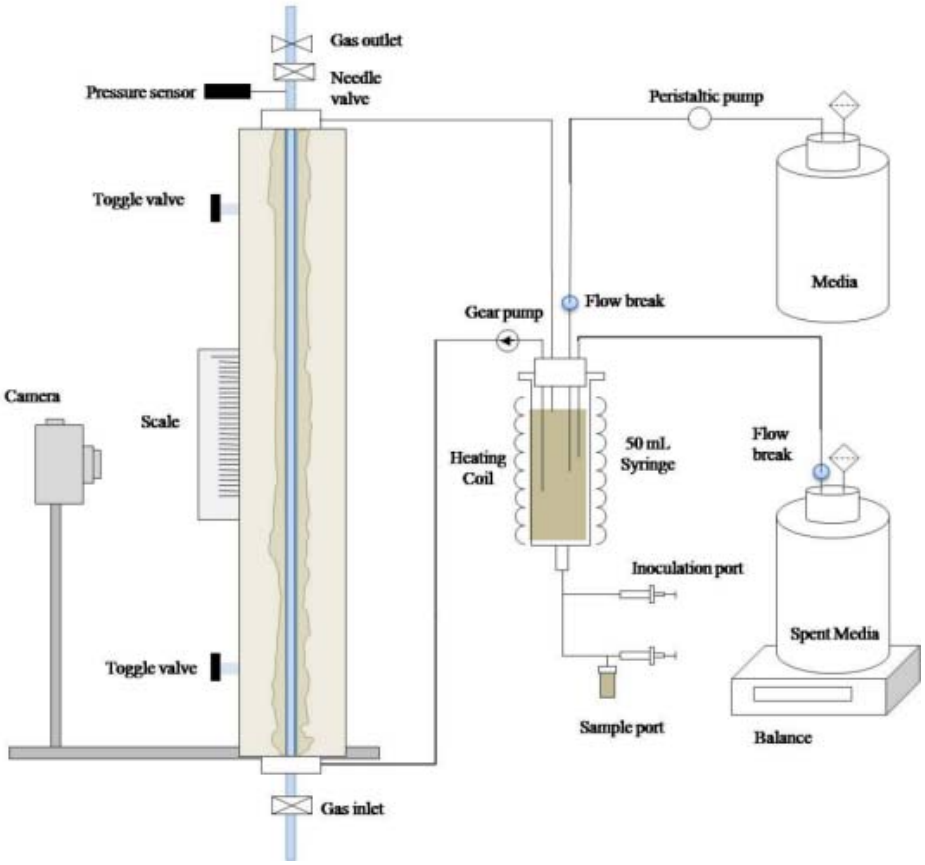
Supplemental figure 1 Light microscopy images of cover slips taken with 20 x magnification from a 6-well plate experiment, stained with crystal violet and sampled after 10 h (A), 24 h (B), 34 h (C), 48 h (D), 58 h (E) and 72 h (F) of incubation in 10 mM 4-fluorobenzoate as a sole source of carbon. The control experiments did not indicate any growth (not shown).



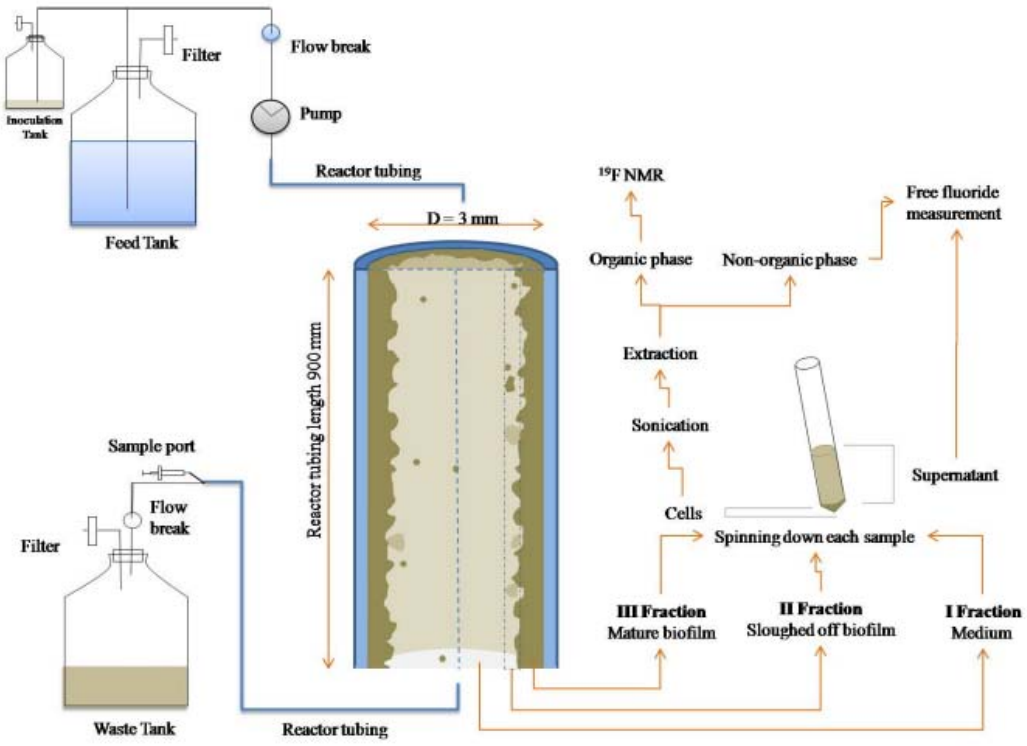
Supplemental figure 2 Light microscopy images of cover slips taken with 20 x magnification from a 6-well plate experiment, stained with crystal violet and sampled after 10 h (A), 24 h (B), 34 h (C), 48 h (D), 58 h (E) and 72 h (F) of incubation in 10 mM benzoate as a sole source of carbon. The control experiments did not indicate any growth (not shown).



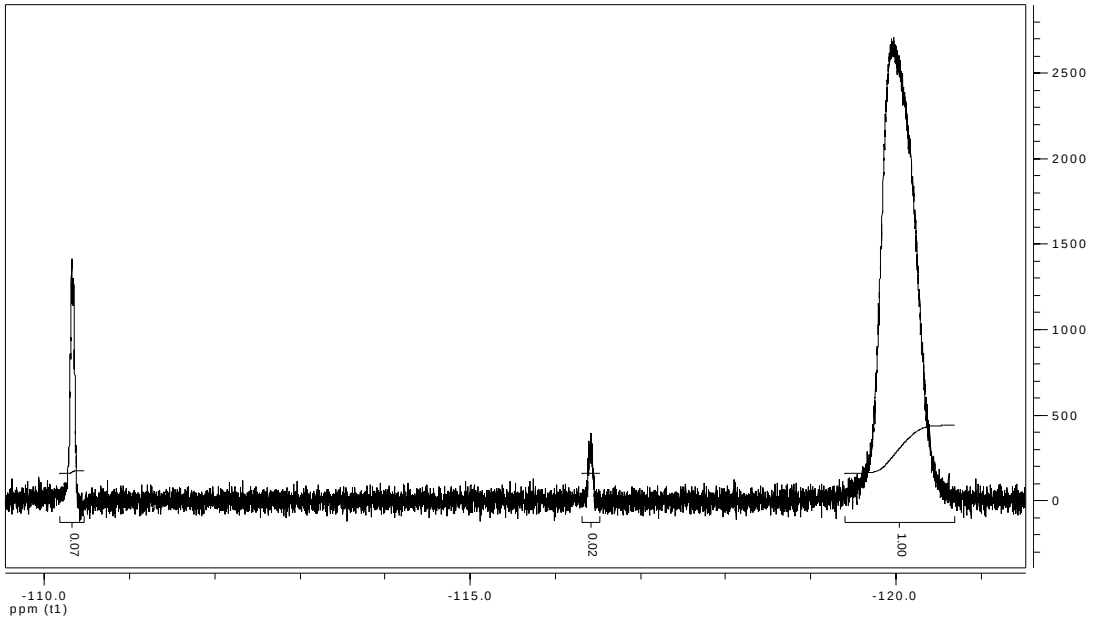
Supplemental figure 3 Light microscopy images of cover slips taken with 20 x magnification from 6-well plate experiments, stained with crystal violet and sampled after 10 h (A), 24 h (B), 34 h (C), 48 h (D), 58 h (E) and 72 h (F) of incubation in 10 mM benzoate as sole source of carbon with 40 mM sodium fluoride addition. The control experiments did not indicate any growth (not shown).



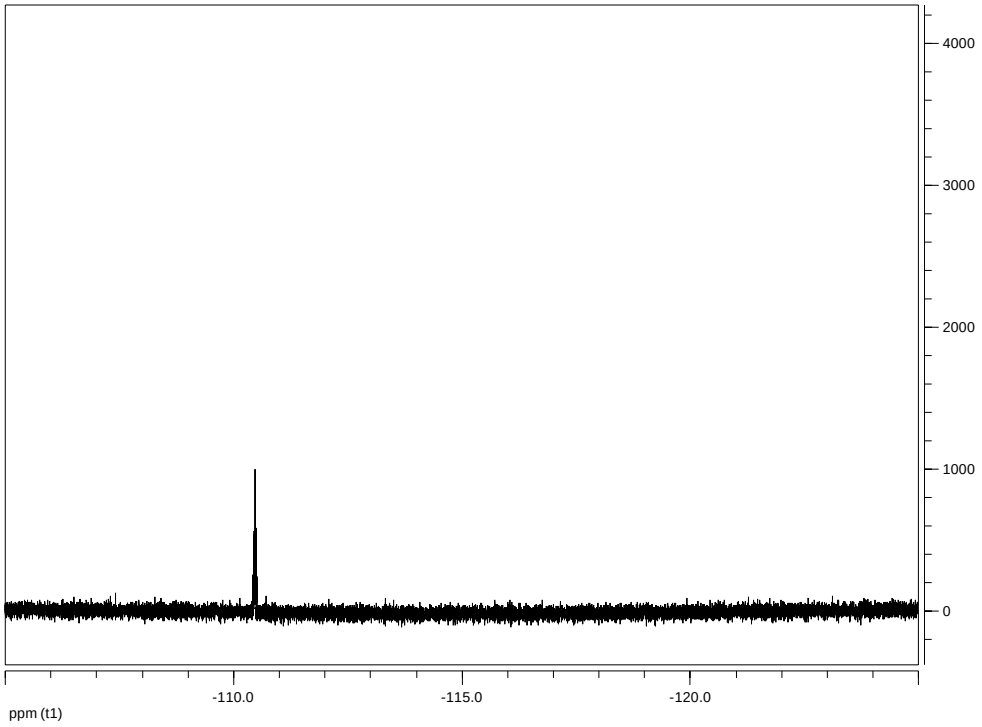
Supplemental figure 4 Schematic of the membrane-aerated bioreactor (MABR).



Supplemental figure 5 Schematic of tubular biofilm reactor (TBR) illustrating the procedure for establishing the fluoride mass balance.



Supplemental figure 6. ^{19}F NMR spectrum of culture supernatant showing resonances of 4-fluorobenzoate (δ -110 ppm), 4-fluorocyclohexadiene-*cis*,*cis*-1,2-diol-1-carboxylate (δ -116 ppm) and fluoride ion (δ -120 ppm).



Supplemental figure 7. ^{19}F NMR spectrum of F3 cell free extract, showing the presence of 4-fluorobenzoate in the biofilm.