An *In Vitro* Test of the Efficacy of an Anti-Biofilm Wound Dressing

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Abstract

Broad-spectrum antimicrobial agents, such as silver, are increasingly being formulated into medicated wound dressings in order to control colonization of wounds by opportunistic pathogens. Medicated wound dressings have been shown *in-vitro* to be effective against planktonic cultures, but *in-vivo* bacteria are likely to be present in biofilms, which makes their control and eradication more challenging. Recently, a functional wound dressing (AQUACEL® Ag+ Extra™ (AAg+E)) has been developed that in addition to silver contains two agents (ethylenediaminetetraacetic acid (EDTA) and benzethonium chloride (BC)) designed to disrupt biofilms. Here, the efficacy of AAg+E is demonstrated using a biofilm model developed in an isothermal microcalorimeter. The biofilm was seen to remain viable in the presence of unmedicated dressing, silver-containing dressing or silver nitrate solution. In the presence of AAg+E, however, the biofilm was eradicated. Control experiments showed that neither EDTA nor BC alone had a bactericidal effect, which means it is the synergistic action of EDTA and BC disrupting the biofilm with silver being bactericidal that leads to the product's efficacy.

Key words:

Staphylococcus aureus; biofilm; isothermal calorimetry; wound dressings; silver

1. Introduction

The process of healing of chronic cutaneous wounds is complex and may be affected by the presence of microorganisms (Bowler, 2002). In particular, bacterial contamination may slow wound healing, result in damage to surrounding tissue and ultimately lead to infection of the host (Landis, 2008). Progression to infection may also be aided by other factors including poor blood supply to the wound and the intrinsic virulence properties of the invading organisms (Siddiqui and Bernstein, 2010; Bowler et al, 2001).

The fact that treatment of bacterial infection at the wound site has the potential significantly to reduce the time for wound healing has led to the development of a number of medicated wound dressings containing antimicrobial agents. Silver is a particularly widely used agent, as it shows broad antimicrobial (against both Gram-negative and Gram-positive organisms, Miraftab et al, 2014) and anti-fungal activity (Bowler et al, 2005), although there is debate as to the specific efficacy of silver (Aziz et al, 2012; White and Cutting, 2006) and to its potential toxicity (Hermans, 2006).

An added complexity in the treatment of chronic wound infections is that the organisms are frequently present as biofilms, with this form of bacterial growth being increasingly implicated in cases of poor wound healing (Hurlow and Bowler, 2012). Evidence for biofilm involvement in chronic wounds comes from macroscopic (Hurlow and Bowler, 2012; Metcalf and Bowler, 2013) and microscopic observation of biofilms (James et al., 2008; Metcalf and Bowler, 2013). A study involving *in vivo* wound models of a *Staphylococcus aureus* infection also showed microscopic evidence of biofilm formation along with a demonstration of physiological differences between planktonic cells and biofilm bacteria recovered from wounds (Davis et al. 2008).

While the drivers for biofilm formation are not completely clear, experimentally it is usually the case that microbial biofilms show reduced antimicrobial susceptibility than comparable planktonic organisms (Davis et al, 2008, Percival et al, 2011). Antibiotic resistance has been ascribed to several mechanisms, including the production of

inactivating enzymes, the presence of persister cells and the protective effects of extracellular polymeric substances (EPS) (Smith, 2005).

Successful treatment of chronic wound infections therefore requires development of next generation of medicated dressings that are efficacious against biofilms. AQUACEL® Ag+ Extra™ (AAg+E) is a recently developed dressing that contains two agents that are known to disrupt biofilms, ethylenediaminetetraacetic acid (EDTA) and benzethonium chloride (BC), in addition to ionic silver as an antimicrobial agent. A recent cohort analysis of wound healing data involving 121 clinical cases showed AAg+E to result in progress towards healing in over 90% of wounds which were previously stalled, infected or at risk of infection and with a high suspicion of biofilm contamination (Metcalf et al, 2014).

Determination of wound dressing efficacy using analytical or traditional microbiological methods is tricky, because of the challenges inherent in determining viable bacterial counts in a heterogeneous system. Isothermal microcalorimetry (IMC) is one technique that offers potential in this area, since it can detect the power resulting from bacterial growth without requiring optical clarity of the sample. We showed previously how IMC can be used to quantitate wound dressing efficacy (Gaisford et al, 2009; Said et al, 2014) against planktonic cultures of two common wound pathogens. To use IMC to investigate the efficacy of AAg+E against biofilms, however, requires development of a biofilm model. Hence, the specific aim of this work was to develop a biofilm model suitable for use with IMC and to use the model to explore the efficacy of AAg+E.

2. Material and Methods

Ethylenediaminetetraacetic acid (EDTA), benzethonium chloride (BC) and silver nitrate ($AgNO_3$) were purchased from Sigma (UK) and used as received. Wound dressings, AQUACEL® (AH), AQUACEL® Ag (AAgH) or AQUACEL® Ag Extra (AAg+E) were supplied by ConvaTec Ltd. The wound dressings, all comprised of

101 sodium carboxymethylcellulose fibres, differ in that AH has no antimicrobial agent
102 while AAgH contains ionic silver and AAg+E contains ionic silver, EDTA and BC.
103 The challenge organism, *Staphylococcus aureus* NCIMB 9518, was grown overnight
104 in nutrient broth (NB; Oxoid Ltd) for 16h at 37 ^{o}C. Cells were then harvested, washed
105 in phosphate buffered saline (PBS), resuspended in 15 %v/v glycerol at an organism
106 density of 10^{8} cfu/ml and frozen in aliquots (1 mL) over liquid nitrogen (Beezer et al,
107 1976; Cosgrove, 1979). Aliquots were stored under liquid nitrogen until required.
108 Previous experience (data not shown) has indicated that organisms can be stored for
109 over 6 years in this frozen state and remain viable post thawing with less than 1%
110 decrease in viability.
111 Ampoules were prepared by adding molten agar (300 µL, Agar No. 3, Oxoid), which
112 was allowed to set. Tryptone soya broth (TSB, 1 mL) was added to prepared
113 ampoules and inoculated with *S. aureus* to a final population density of $1x10^{6}$ cfu/mL.
114 The ampoules were then sealed and incubated at 37 ^{o}C with agitation for 24 hours.
115 Thereafter, the ampoules were opened and the TSB was carefully poured out. The
116 ampoules were gently rinsed with sterile distilled water a minimum of five times to
117 ensure effective removal of planktonic cells. Fresh, sterile TSB (1 mL) and where
118 required wound dressing (3.3 mg), EDTA (0.14% w/v), BC (0.4% w/v) or $AgNO_3$ (1 x
119 10^{-4} M) was then added to the ampoules, which were hermetically sealed and placed
120 into calorimeters.
121 Cell counts were determined after each calorimetric experiment. After removal of
122 TSB and extensive rinsing, as above, fresh PBS was added to ampoules, which were
123 then sealed. Ampoules were then briefly vortexed before being sonicated for a period
124 of 5 minutes. Colony counts were determined by serially diluting the PBS from the
125 ampoules and spread plating onto iso-sensitest agar. Colonies were counted
126 following 16h incubation at 37 ^{o}C.
127 Calorimetric data were recorded with a 2277 Thermal Activity Monitor (TAM; TA
128 Instruments Ltd, UK) operated at 37 ^{o}C. Data capture was initiated exactly 30 min

post inoculation with the dedicated software package Digitam 4.1 (1 data point every 10 s, amplifier setting 300 μW). The instrument was calibrated periodically by the electrical substitution method. Data were analysed using Origin 8.1 (Microcal Software Inc).

3. Results and Discussion

Although not discussed here, it was not possible to culture a biofilm directly onto the glass walls of the ampoule. Rather, the agar layer was necessary to encourage biofilm attachment and growth. A similar effect has been noted in ecological research where a twofold increase in the accumulation of diatoms (phytoplanktonic algae) was seen on a surface when unenriched agar was added (Stevenson, 1983). The rationale in that study was the use of agar to 'simulate mucilage of immigrating organisms', in other words to create a 'slimy', polymeric conditioning film. In relation to *in vivo* correlation it has been suggested that the use of agar simulates a microbial colonisation of a moist surface, similar to wounds (Daeschlein, 2010).

The physiology of cells in biofilms is distinct from those in planktonic culture because of their sessile nature and differences in exposure to nutrients and gases in their microenvironment (Stewart and Franklin, 2008). Diffusion is the main mechanism of delivery of solutes to biofilm clusters (Stewart, 2003) and so concentration gradients exist that depend on the thickness of the biofilm. It follows that delivery of antimicrobial agents will similarly be rate limited by this diffusion mechanism, and their efficacy may be reduced relative to planktonic cultures (up to a 1000 fold decrease in susceptibility has been reported, Stewart and Costerton, 2001).

Similarly, the power produced by an established biofilm will differ from that of a planktonic culture. As has been discussed in the literature (Braissant et al, 2010; Said et al, 2014) the power output from planktonic cultures will show a series of exothermic peaks and troughs with time as the organisms utilise the available nutrients to increase in number. Integration of the power data with respect to time

gives a plot of cumulative heat versus time, which is comparable to the standard growth curves determined with optical density (OD) readings.

Typical power-time data for *S. aureus* biofilms are shown in Figure 1. Biofilms were found not to give as repeatable data as planktonic cultures. Similar variability was seen in a study by Astasov-Frauenhoffer et al (2012). However, the degree of variability was small enough to permit study of the efficacy of the dressing. It is apparent that there is an exothermic peak initially, followed by a smaller, but persistent, exothermic power. It is assumed that the initial peak represents either growth of planktonic cells released from the biofilm or growth and metabolism within the biofilms themselves. The power then resolves to a non-zero baseline, which is assumed to be associated with biofilm metabolism.

The presence of the initial exotherm is surprising. Following 24 hours incubation, the putative biofilm samples were extensively rinsed in order to remove planktonic cells. Each rinsing can be considered as a 1:1 dilution as the volume of diluent used was equivalent to that of the original growth medium in the culture. As such, very little or no power, or at least a significant delay in onset of power signals, would be expected from planktonic cells as these would have been diluted down to undetectable levels (given that the lower limit of detection for IMC is approximately $1x10^5$ – $1x10^6$ cfu/mL, Said et al, 2014).

Cell counts for biofilm samples suggest a potential explanation. Immediately following preparation (i.e. before being loaded into the calorimeter) the average cell count was $4x10^7$ cfu/mL. After 24 h in the calorimeter, the average cell count had increased to $1x10^8$ cfu/mL. This suggests that cell division continues within the biofilm, presumably until the available nutrients and/or gases in the fresh medium are exhausted, after which the system reverts to a stasis metabolism. An alternative explanation is that since rinsing will disturb the isotonic balance of the system, the biofilm responds by dispersing bacteria from the film as a survival response. These bacteria then grow as a planktonic population.

185 The corresponding cumulative heat plot for the biofilm is shown in Figure 2 (all further
186 data are shown as cumulative heat plots for familiarity and ease of comparison with
187 OD data).
188 Previously (Said et al, 2014) we showed that $AgNO_3$ at a concentration of 1×10^{-4} M
189 was bactericidal against planktonic *S. aureus*. Figure 3 shows the growth curves for
190 planktonic and biofilm cultures in the presence of this silver concentration. As
191 expected, no growth is seen in planktonic culture, but the biofilm culture shows
192 growth following an initial delay. The average cell count following the calorimetric
193 experiment was 1.5×10^7 cfu/mL, not much lower than that recorded for the
194 untreated biofilm, which confirms that biofilm formation confers on the participant
195 organisms a significant degree of protection against antimicrobial agents.
196 The implication of this finding is that a wound dressing containing silver alone might
197 be less effective against a biofilm culture, and this effect is indeed observed
198 experimentally, Figure 4. Dressing alone (AH) did not inhibit growth, while silver-
199 containing dressing (AAgH) only delayed growth (the same effect noted above in the
200 silver nitrate experiments).
201 Successful eradication of an established biofilm requires delivery of agents to disrupt
202 biofilm structure in addition to an antimicrobial compound. This is the formulation
203 strategy behind AAg+E and the data in Figure 4 show a significant reduction in
204 growth with this dressing. The average cell count following exposure to AAg+E was 2
205 $\times 10^4$ cfu/mL, so the dressing can be deemed to be bactericidal in nature because it
206 induced a 3 log reduction in viable cells relative to the control.
207 The question then remains as to the relative contributions of the components in
208 AAg+E to the overall efficacy of the dressing. Biofilms were exposed to solutions
209 containing only $AgNO_3$, only EDTA and BC or all three components (Figure 5). In all
210 cases a small amount of AH (i.e. plain dressing) was included, the intention being to
211 imitate the action of a dressing containing only these components. It is apparent that
212 EDTA and BC cause an initial delay but then growth is seen which is identical to the

213 control. We noted previously that the presence of AH can alter the kinetics of growth
214 (Said et al, 2014), possibly because the hydrofiber network can sequester planktonic
215 bacteria, changing the rate of supply of nutrients and altering local pH. It is also
216 possible that the hydrofiber itself can cause physical disruption of a biofilm. An effect
217 on growth rate in the presence of AH is seen in Figure 4. (Silver nitrate is seen to
218 inhibit growth for at least 10h, after which growth similar to the control is seen. In
219 neither case therefore do these components act to inhibit the biofilm. When they are
220 added in combination, however, a reduction in growth is seen, which confirms the
221 hypothesis that it is the combination of biofilm disrupters and an antimicrobial agent
222 working in synergy that is necessary for efficacy.
223 The effect of EDTA has been attributed to its properties as a metal chelator, since the
224 chelation of divalent cations that are important in ensuring the structural integrity of
225 cells causes disruption and prolonged exposure may result in a biocidal effect (Banin
226 et al., 2005). Certain divalent cations have also been indicated in the structural
227 integrity of the biofilm EPS matrix (Banin et al., 2005; Percival et al., 2005) and
228 sequestration of these cations may result in dispersal of the biofilm.
229 EDTA may also play a role in the delivery of silver to the bacteria. Since silver is
230 contained in the dressing in its ionic form, delivery to its site of action will be affected
231 both by the concentration of free silver in solution and the relative association
232 constants between silver and components in solution and the bacterial cell wall. It is
233 likely that binding to EDTA affects the relative positions of these association
234 constants, and since the silver appears to have faster action in the presence of EDTA
235 the data imply the association constant of silver with EDTA is fast, ensuring silver
236 ions are rapidly removed from the dressing, but not as strong as the association
237 constants of silver with the cells.
238 BC, on the other hand, is a surfactant. In high concentrations BC has antiseptic and
239 anti-infective properties that have been shown to have anti-biofilm activity against
240 oral fungal biofilms (Ichikawa et al., 2008), but the concentrations achieved from

241 AAg+E are below the MIC. Thus, BC acts to reduce surface tension, affecting biofilm
242 architecture and cell-cell interactions (Davey et al., 2003), promoting the activity of
243 silver when delivered with EDTA.

244 **4. Summary**

245

246 A biofilm model was developed that enabled the use of IMC to test the efficacy of an
247 anti-biofilm wound dressing. It was shown that a broad spectrum antimicrobial agent
248 alone was not effective against the biofilm but that when biofilm disrupting agents
249 were included in the dressing bactericidal action was seen.

250

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257 **References**

258 Astasov-Frauenhoffer, M., Braissant, O., Hauser-Gerspach, I., Daniels, A.U., Weiger,
259 R., Waltimo, T., 2012. Isothermal microcalorimetry provides new insights into biofilm
260 variability and dynamics. FEMS Microbiol. Lett., 337, 31-37.
261
262 Aziz, Z., Abu, S.F., Chong, N.J., 2012. A review of silver-containing dressings and
263 topical silver agents (used with dressings) for burn wounds. Burns, 38, 307-318.
264
265 Banin, E., Brady, K.M., Greenberg, E.P., 2006. Chelator-induced dispersal and killing
266 of *Pseudomonas aeruginosa* cells in a biofilm. App. Environ. Microbiol. 72, 2064-
267 2069.
268
269 Beezer, A.E., Newell, R.D., Tyrrell, H.J.V., 1976. Application of flow microcalorimetry
270 to analytical problems – preparation, storage and assay of frozen inocula of
271 saccharomyces cerevisiae. J. Appl. Bact. 41, 197-207.
272
273 Bowler, P.G., 2002. Wound pathophysiology, infection and therapeutic options.
274 Annals Med. 34, 419-427.
275
276 Bowler, P.G., Duerden, B.I., Armstrong, D.G., 2001. Wound microbiology and
277 associated approaches to wound management. Clin. Microbio. Rev. 14, 244-269.
278
279 Bowler, P.G., Jones, S.A., Walker, M., Parsons, D., 2005. Microbicidal properties of a
280 silver-containing Hydrofiber® dressing against a variety of burn wound pathogens. J.
281 Burn Care Rehabil. 25, 192-196.
282
283 Braissant, O., Wirz, D., Göpfert, B., Daniels, A.U., 2010. Use of isothermal
284 microcalorimetry to monitor microbial activities. FEMS Microbio. Lett. 303, 1-8.

285

286 Cosgrove, R.F., 1979. Long-term storage of microorganisms used in antimicrobial
287 effectiveness tests. J. Assoc. Off. Anal. Chem. 62, 1188-1190.
288

289 Daeschlein, G., Von Woedtke, T., Kindel, E., Brandenburg, R., Weltmann, K.D.,
290 Jünger, M., 2010. Antibacterial activity of an atmospheric plasma jet against relevant
291 wound pathogens in vitro on a simulated wound environment. Plasma Process. Poly.
292 7, 224-230.
293

294 Davey, M.E., Caiazza, N.C., O'Toole, G.A., 2003. Rhamnolipid surfactant production
295 affects biofilm architecture in *Pseudomonas aeruginosa* PA01. J. Bacterio. 185,
296 1027-1036.
297

298 Davis, S.C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W.H., Mertz, P.M., 2008.
299 Microscopic and physiologic evidence for biofilm-associated wound colonization *in*
300 *vivo*. Wound Repair Regen. 16, 23-29.
301

302 Gaisford, S., Beezer, A.E., Bishop, A.H., Walker, M., Parsons, D., 2009. An in vitro
303 method for the quantitative determination of the antimicrobial efficacy of silver-
304 containing wound dressings. Int. J. Pharm. 366, 111-116.
305

306 Hermans, M.H., 2006. Silver-containing dressings and the need for evidence. Am. J.
307 Nursing 106, 60-68.
308

309 Hurlow, J., Bowler, P.G., 2012. Potential implications of biofilm in chronic wounds: a
310 case séries. J. Wound Care 21, 109-119.
311

312 Ichikawa, T., Yano, Y., Fujita, Y., Kashiwabara, T., Nagao, K., 2008. The
313 enhancement effect of three sugar alcohols on the fungicidal effect of benzethonium
314 chloride toward *Candida albicans*. J. Dentistry 36, 965-968.
315
316 James, G.A., Swogger, E., Wolcott, R., Pulcini, E., Secor, P., Sestrich, J., Costerton,
317 J.W., Stewart, P.S., 2008. Biofilms in chronic wounds. Wound Repair Regen. 16, 37-
318 44.
319
320 Landis, S.J., 2008. Chronic wound infection and antimicrobial use. Ad. Skin Wound
321 Care 21, 531-540.
322
323 Metcalf, D.G., Bowler, P.G., 2013. Biofilm delays wound healing: A review of the
324 evidence. Burns & Trauma, 1, 5-12
325
326 Metcalf, D.G., Walker, M., Mathison, R., 2014. A cohort analysis of wound healing
327 data from evaluations of a next-generation antimicrobial dressing. Abstracts of the
328 24th Conference of the European Wound Management Association, P291.
329
330 Miraftab, M., Masood, R., Edward-Jones, V., 2014. A new carbohydrate-based
331 wound dressing fibre with superior absorption and antimicrobial potency. Carb. Pol.
332 101, 1184-1190.
333
334 Percival, S.L., Hill, K.E., Malic, S., Thomas, D.W., Williams, D.W., 2011. Antimicrobial
335 tolerance and the significance of persister cells in recalcitrant chronic wound biofilms.
336 Wound Repair Regen. 19, 1-9.
337

338 Percival, S.L., Kite, P., Eastwood, K., Murga, R., Carr, J., Arduino, M.J., Donlan,
339 R.M., 2005. Tetrasodium EDTA as a novel central venous catheter lock solution
340 against biofilm. Infec. Cont. Hosp. Epidem. 26, 515-519.
341
342 Said, J., Dodoo, C.C., Walker, M., Parsons, D., Stapleton, P., Beezer, A.E., Gaisford,
343 S., 2014. An *in vitro* test of the efficacy of silver-containing wound dressings against
344 *Staphylococcus aureus* and *Pseudomonas aeruginosa* in simulated wound fluid. Int.
345 J. Pharm. 462, 123-128.
346
347 Siddiqui, A.R., Bernstein, J.M., 2010. Chronic wound infection: Facts and
348 controversies. Clinics in Dermatology, 28, 519-526.
349
350 Smith, A.W., 2005. Biofilms and antibiotic therapy: Is there a role for combating
351 bacterial resistance by the use of novel drug delivery systems? Ad. Drug Del. Rev.
352 57, 1539-1550.
353
354 Stevenson, R.J., 1983. Effects of current and conditions simulating autogenically
355 changing microhabitats on benthic diatom immigration. Ecology 64, 1514-1524.
356
357 Stewart, P.S., 2003. New ways to stop biofilm interactions. The Lancet 361, 97.
358
359 Stewart, P.S., Costerton, W.J., 2001. Antibiotic resistance of bacteria in biofilms. The
360 Lancet 358, 135-138.
361
362 Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nat. Rev.
363 Microbiol. 6, 199-210.
364

White, R., Cutting, K., 2006. Exploring the effects of silver in wound management – What is optimal? Wounds – A Compendium of Clinical Research and Practice. 18, 307-314.

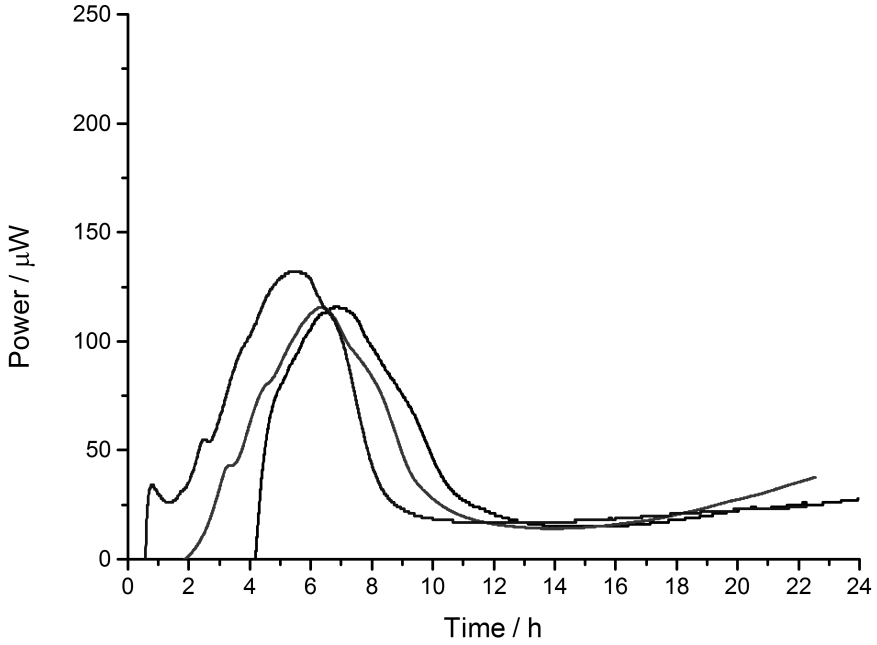
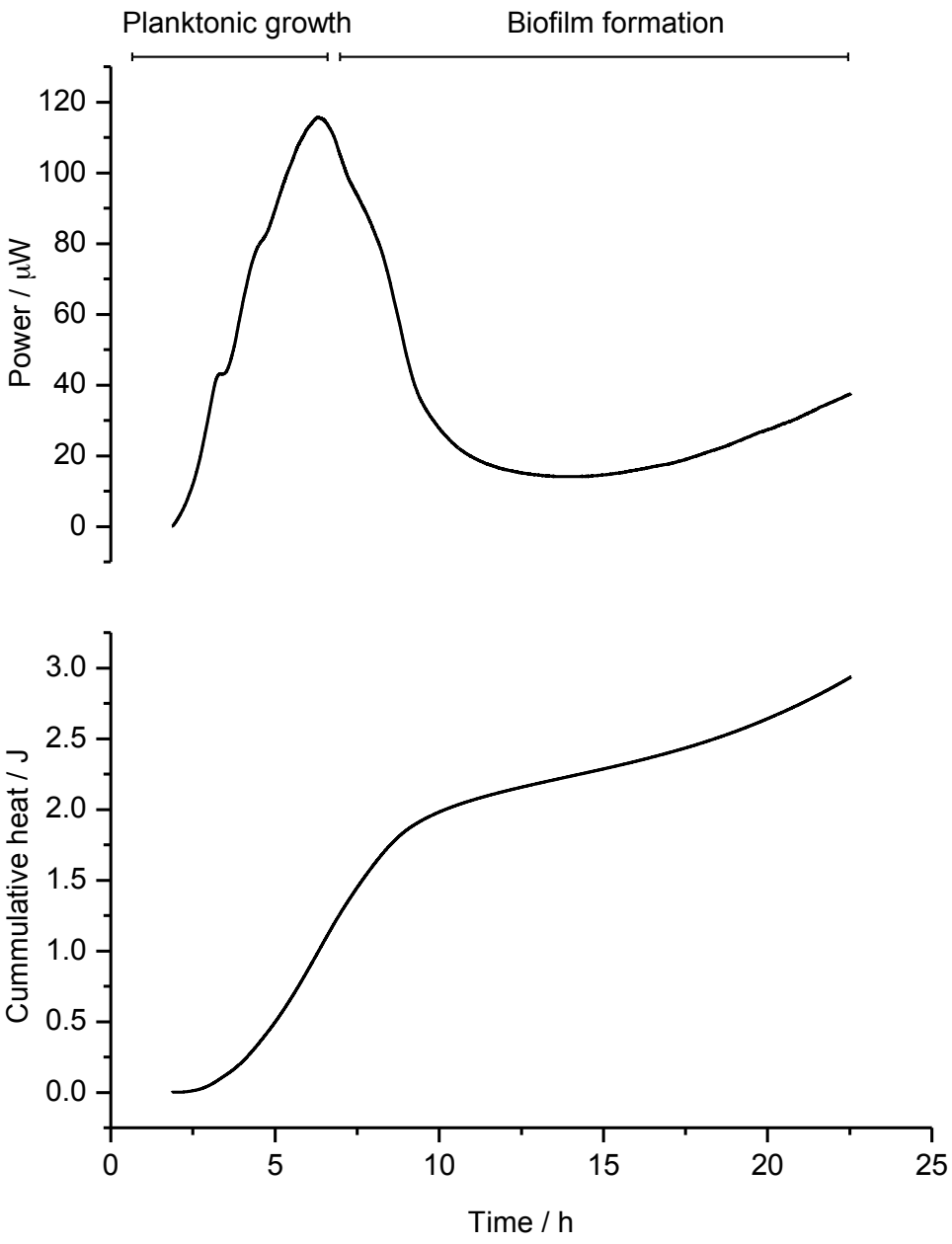


Figure 1: Power-time data for biofilms grown on agar, showing the level of reproducibility for three samples.



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377 **Figure 2: Power-time data for the control biofilm (bottom) and corresponding**

378 **growth curve (top)**

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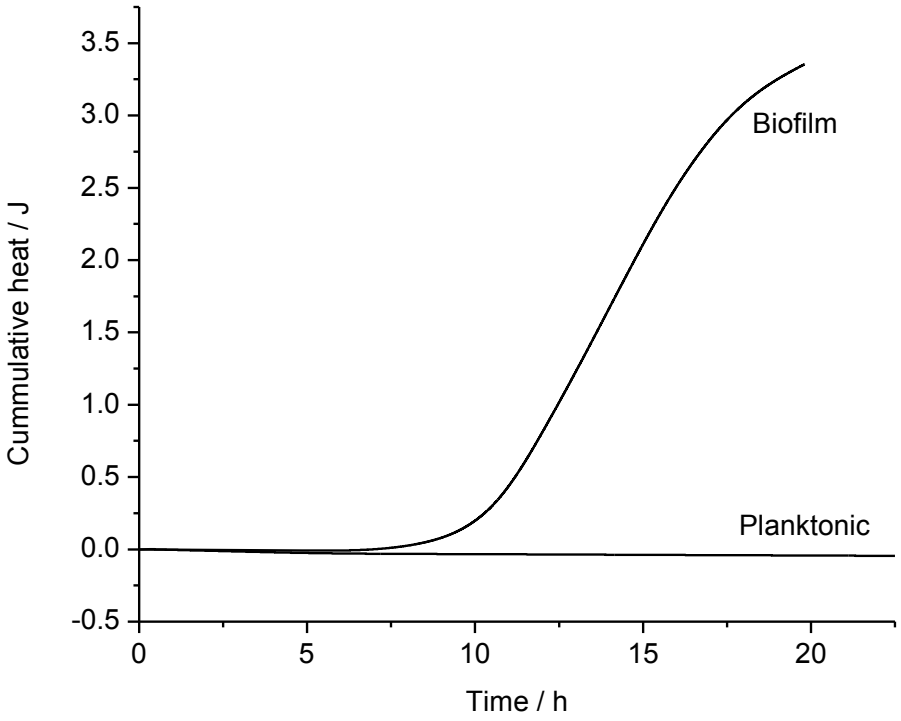

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383 **Figure 3: Growth curves for planktonic and biofilm cultures in the presence of**

384 **1 x 10^{-4} M $AgNO_3$.**

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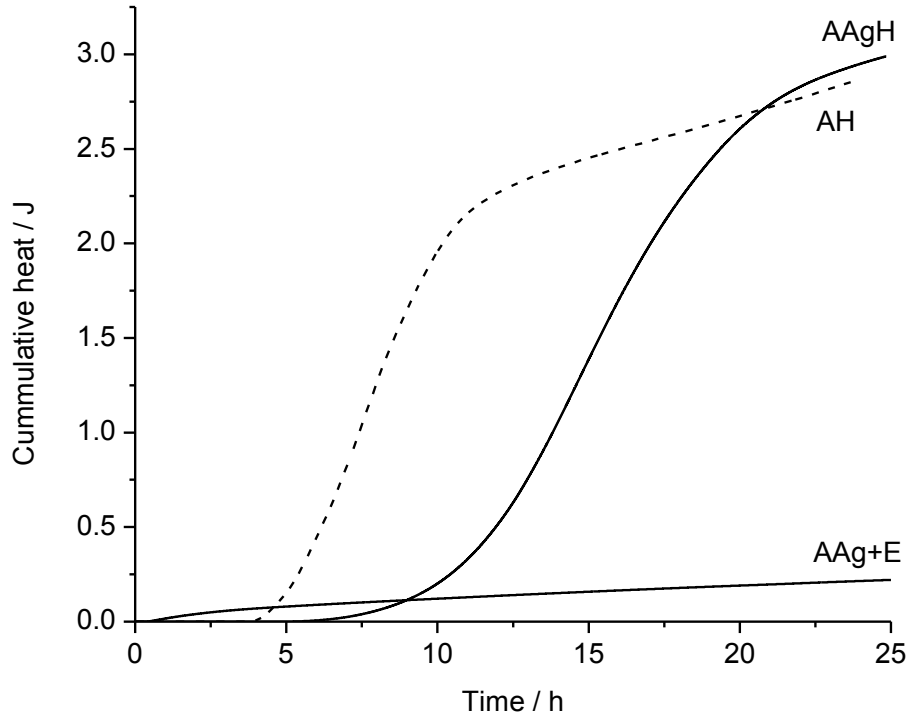

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388 **Figure 4: Growth curves for biofilms in the presence of AH, AAgH and AAg+E.**

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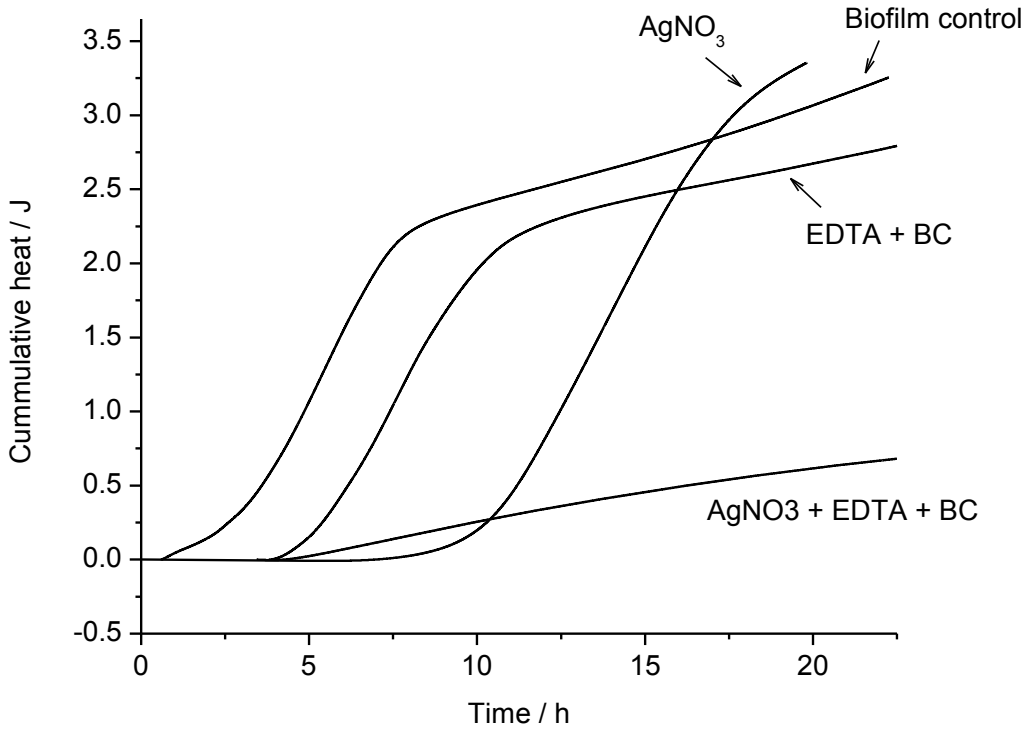

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392 **Figure 5: Growth curves for biofilm alone and in the presence of $AgNO_3$, EDTA**

393 **+ BC and $AgNO_3$ + EDTA + BC.**

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