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## **Ethidium bromide efflux by: modulation by metabolic energy, pH, ions and phenothiazines**

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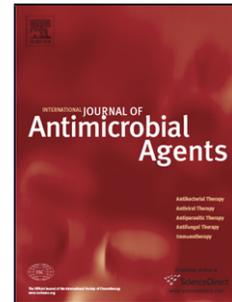
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31 **Abstract**

32 The main efflux pump of Salmonella Enteritidis serovar typhimurium that obtains its energy for  
33 the extrusion of a noxious agent from the proton motive force (PMF) has been studied with the  
34 aid of the Ethidium Bromide semi-automated method under conditions which define the role that  
35 metabolic energy, ions and pH play in the extrusion of the universal substrate ethidium bromide  
36 (EB). The results obtained in this study indicate that in minimal medium containing sodium,  
37 whereas at pH 5, efflux of EB is independent of glucose, at pH 8, metabolic energy is an absolute  
38 requirement for the maintenance of efflux. In deionised water pH 5.5, metabolic energy is  
39 required for maintenance of efflux. The inhibitory effect of the ionophore carbonyl cyanide *m*-  
40 chlorophenylhydrazone (CCCP) on efflux is shown to be minimised by low pH, and at high pH,  
41 by metabolic energy. Similarly, thioridazine, an inhibitor of metabolic enzymes inhibits efflux of  
42 EB only at pH 8 and the degree of inhibition lessened by the presence of metabolic energy.

43  
44 **Keywords:** proton motive force; hydronium ions, Salmonella Enteritidis, efflux pump; pH;  
45 metabolic energy; ions, CCCP, thioridazine.

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## 54 **1. Introduction**

55 Infections caused by food-borne salmonella continue to manifest themselves even in well  
56 developed western countries where control over the preparation of food is unquestionably of high  
57 quality (1). Moreover, because many of these foods are meats that have been obtained from  
58 animals that have been treated for prolonged periods with tetracycline, and prolonged exposure of  
59 Gram-negative bacteria to this antibiotic causes the development of multi-drug resistant  
60 phenotypes (2-4), there is the distinct possibility that meats may not only be contaminated with  
61 salmonella or other coliform bacteria, but with MDR phenotypes of these bacteria (5).

62  
63 The MDR phenotype of Gram-negative bacteria is mainly due to over-expressed efflux pumps  
64 which extrude members from two or more antibiotic classes before the antibiotics can reach their  
65 intended targets (6). The AcrAB-TolC efflux pump is the main MDR efflux pump of salmonella  
66 (6) and belongs to the genetically defined class resistance nodular division family (RND). RND  
67 efflux pumps derive the energy for activity from the proton motive force (PMF) which can be  
68 collapsed by exposure of the bacterium to cyanide m-chlorophenylhydrazine (CCCP) (7).

69  
70 The components that make up the AcrAB-TolC efflux pump of salmonella are: i) the trimeric  
71 transporter protein AcrB that is attached to the inner membrane (plasma membrane) and  
72 recognizes and binds the substrate that is present in the periplasm, or within outer leaflet of the  
73 plasma membrane, or in the cytoplasm (8), and rapidly transfers the substrate to ii) the TolC  
74 component which is a conduit to the surface of the cell. The third component of the AcrAB-TolC  
75 efflux pump are two fusion proteins AcrA which bridge the joining of the AcrB to TolC and

76 which have been suggested to assist the extrusion process by a “peristaltic” type mechanism  
77 (9,10).

78  
79 Ingested salmonella must survive the acid rich environment of the duodenum which receives bile  
80 salts from the liver that are toxic to the organism (11). When the organism reaches the colon, the  
81 pH is near neutral but the presence of bile salts still presents a toxic situation which must be dealt  
82 with for the entire duration of its colonic presence. The means by which the organism escapes  
83 injury from bile salts is by the activity of its AcrAB-TolC efflux pump which extrudes the  
84 noxious compounds prior to their reaching their intended targets (11). One may suppose that as  
85 long as the organism resides in the colon, the activity of the AcrAB-TolC efflux pump also  
86 protects it from antibiotics, and therefore, this would be the major reason as to why therapy of  
87 salmonellosis is problematic.

88  
89 Recent studies have shown that the dissociation constant ( $K_d$ ) for a substrate bound to the AcrB  
90 transporter is markedly affected by pH (12). Whereas at low pH of 5 the  $K_d$  is highest, with  
91 increasing pH the  $K_d$  decreases to a point where dissociation is almost absent (pH 8.5). If the  
92 AcrB transporter is to function, the dissociation of the substrate must be high, and if not, the  
93 transport function would slow down, thereby compromising the existence of the organism when  
94 present in an environment containing a noxious agent (bile salts, antibiotics). Because the  
95 AcrAB-TolC efflux pump is in reality a “tube” whose open terminal end (TolC channel) provides  
96 exposure of the transporter to the pH of the milieu, one would expect that when the salmonella  
97 organism is in the duodenum where the pH is lower than pH 5, dissociation of the bile salt or  
98 antibiotic from the site of transporter to which it was initially bound, would be rapid. At pH 8 the

99 dissociation of the substrate (bile salt or antibiotic) from the AcrB transporter would be slow.  
100 Because the organism survives in the colon (*ca.* pH 7) where the concentration of bile salts is  
101 high, the dissociation of the substrate from the transporter must be rapid if the efflux pump is to  
102 protect the organism. How does the organism accomplish this in a pH milieu where dissociation  
103 of the substrate from the transporter is slow?.

104

105 The study to be described evaluates the *in vitro* effect of varying pH on the efflux of the universal  
106 efflux pump substrate-ethidium bromide (EB), a fluorochrome that is used for the assessment of  
107 efflux via an automated method (13), the modulation of efflux by metabolic energy, and the role  
108 of ions in efflux.

109

110

111 **2. Materials & Methods.**

112 *2.1 Bacteria Salmonella Enteritidis NCTC 13349.*

113 *2.2 Materials*

114 Media employed in this study for maintaining Salmonella Enteritidis NCTC 13349: Mueller-

115 Hinton (MH) in powder form purchased from Difco for the preparation of broth and agar;

116 ethidium bromide (EB), phosphate buffered sodium chloride (PBS), sodium chloride, glucose,

117 sodium pyruvate, ethanol, ATP, carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) thioridazine

118 (TZ) and orthovanalate (OV) purchased from Sigma-Aldrich Química SA (Madrid Spain).

119 Sodium Chloride purchased from Aldrich.

120

121 *2.3 Minimum inhibitory concentrations (MICs) of EB, CCCP, TZ and OV performed in MH as*

122 *per CLSI guidelines (14). MICs were determined for each of these agents in order to select*

123 *concentrations that had no effect on the viability of the bacterium.*

124

125 *2.4 Real-time accumulation of EB*

126 was performed using our previously developed semi-automated method (11, 12). Briefly, the

127 study strain was cultured in MH broth reaching an optical density (OD<sub>545</sub>) of 0.6, the tubes

128 centrifuged at 13,000 rpm for 3 minutes and the supernates discarded. The recovered pellets re-

129 suspended in phosphate buffered saline (PBS) pH 7.4, and centrifuged as before. The final

130 washed pellet was then re-suspended in tubes containing PBS buffered to different pH ranging

131 from 5 through 8 or deionised water. The final OD<sub>545</sub> was adjusted to 0.6.

132

133 To 100  $\mu\text{l}$  tubes a volume of 50  $\mu\text{l}$  containing PBS at varying pH or deionised water to match that  
134 of the initial cell stock with and without glucose to yield a final concentration per ml of 0.6 % or  
135 other sources of metabolic energy, and 1 mg/L of EB. 50  $\mu\text{l}$  of the cell suspension maintained in  
136 varying pH of PBS or deionised water was added to the phosphate-saline pH matching tubes that  
137 lacked or contained metabolic energy or deionised water that lacked or contained metabolic  
138 energy. Similarly, agents such as CCCP, TZ and OV in pH matched PBS or deionised water were  
139 added. Tubes were placed into the Corbett 3000 PCR Thermocycler that had been programmed  
140 for temperature of 37°C, length of cycle in minutes, total number of cycles and for the identity of  
141 each tube with respect to its contents. The amount of relative fluorescence calculated by the  
142 Corbett 3000 instrument is a measure of accumulated EB within the bacterial population (15).  
143 Accumulation assays were repeated by three different co-authors and the qualitative results  
144 obtained were similar.

145

#### 146 *2.5 Assessment of real-time efflux of EB*

147 In order that efflux is assessed, sufficient accumulation of EB must first be established.  
148 Therefore, conditions which maximize accumulation of EB (identified in the text and its figures)  
149 were employed and accumulation of EB at 37°C allowed for a specific period of time after which  
150 the instrument was stopped, various reagents that would allow definition of their effects on efflux  
151 were then added in a volume of 10  $\mu\text{l}$  (controls received 10  $\mu\text{l}$  of matching pH), the instrument re-  
152 started and any change in fluorescence recorded during the remainder of the run. These efflux  
153 assays were repeated by three of the co-authors of this study and the results obtained did not vary  
154 qualitatively.

155

156 **3. Results**

157 The MICs for EB, CCCP, TZ and OV against Salmonella Enteritidis NCTC 13349 are presented  
158 by Table 1. The concentrations of each of these reagents employed in the following sections were  
159 well below  $\frac{1}{2}$  their MICs and had no effect on the viability of the bacterium.

160  
161 The accumulation and efflux of EB by bacteria has been shown to be modulated by pH (16) and  
162 independent of metabolic energy at pH 5 during the assay (15, 16). The effect of pH and energy  
163 source on the accumulation of EB by the NCTC strain maintained in PBS is depicted by Figure 1.  
164 As noted by Figure 1A accumulation of EB in the absence of glucose is markedly affected by pH.  
165 In the presence of glucose during the course of the assay, as noted by Figure 1B, no accumulation  
166 takes place at pH 5; a little accumulation of EB takes place at pH 7.4 and slightly more at pH 8.  
167 These results suggest that accumulation of EB by the NCTC strain, as is the case with other  
168 Gram-negative bacteria (15, 16), is affected by pH and that at pH of 7.4 or greater, glucose plays  
169 a major role in reducing the amount of EB accumulated. That indeed metabolic energy plays a  
170 role in the control of EB accumulation, replacement of glucose with ethanol or pyruvate provides  
171 the needed energy to maintain accumulation at a lower level as opposed to the absence of  
172 metabolic energy alone (data not shown). However, as is the case for glucose, the need for  
173 metabolic energy is greatest with increase of pH. At pH 5, metabolic energy is not needed during  
174 the assay for maintaining a low level of EB accumulation and no additional decrease of EB  
175 accumulation was observed in the presence of pyruvate, glucose or ethanol (data not shown). At  
176 pH 7.4 and pH 8 replacement of glucose with ethanol or pyruvate reduces accumulation.

177

178 Conditions which promote the accumulation of the substrate are considered to be those that  
179 modulate efflux (2, 6, 13, 15-17). Nevertheless, in order that such conditions are identified, an  
180 efflux assay that describes extrusion of the substrate must be conducted. As shown by Figure 2,  
181 in PBS of pH 8, efflux by the salmonella strain takes place only when metabolic energy (glucose,  
182 ethanol, pyruvate) is present in the medium during the duration of the assay. At pH 5, efflux takes  
183 place readily in the absence of any metabolic energy (data not shown).

184  
185 CCCP, a proton ionophore, causes the collapse of the proton motive force (PMF) and hence, deny  
186 protons needed for the activity of an efflux pump system dependent upon the PMF (6, 18). If low  
187 pH is sufficient to maintain efflux, the possibility that at low pH CCCP would have a much  
188 smaller effect on efflux than at high pH, was investigated. As shown by Figure 3, at a pH of 5 the  
189 effect of CCCP on accumulation of EB is much less than at pH 8 when glucose is present.  
190 Similarly, the effect of CCCP on the efflux of EB is much less at pH 5 than at pH 8 (data not  
191 shown). Whereas the lowest concentration of CCCP that promoted accumulation and inhibited  
192 efflux at pH 5 was 2.5 mg/L at pH 8 concentrations of CCCP as low as 1 mg/L produced  
193 significant accumulation and inhibition of efflux at pH 8 (data not shown).

194  
195 Phenothiazines such as thioridazine (TZ) are known to inhibit enzymes involved in metabolism  
196 (19). Because metabolic energy is required for efflux of EB as shown in this study, the effect of  
197 TZ on accumulation and efflux was studied, and the results depicted by Figure 4. Briefly, TZ  
198 inhibits efflux at pH 8 despite the presence of glucose in the system and has less of an effect at  
199 pH 5. Similar results have been obtained with *Escherichia coli* (20).

200 OV is known to affect the activity of metabolic enzymes that furnish energy (ATP) for the  
201 activity of ABC transporters (21). Because ABC transporters hydrolyse ATP subsequent to their  
202 binding the substrate, reduction of ATP levels reduces efflux of the substrate. At pH 8 the  
203 hydrolysis of ATP by ATP synthase is favoured (22-24). In order to determine whether the  
204 marked inhibition of efflux promoted by TZ at pH 8 is due to an inhibition of an ABC  
205 transporter, the efflux assay was conducted in glucose-saline pH 8 with increasing concentrations  
206 of OV. Concentrations of OV as high as 200 mg/L had no effect on efflux of EB (data not  
207 shown) thereby indicating that the effects of TZ on EB efflux at pH 8 did not involve an ABC  
208 transporter.

209  
210 As shown by the studies so far described, efflux at pH 5 does not require metabolic energy for the  
211 duration of the assay. In our laboratory, deionised water has a consistent pH of 5.5 and therefore  
212 this pH is close to that of the PBS of pH 5. Accumulation and efflux were therefore conducted  
213 in deionised water pH 5.5 with and without energy source. As shown by Figure 5A and Figure  
214 5B, metabolic energy is needed to prevent or reduce accumulation of EB as well as to maintain  
215 efflux of EB. Therefore, pH alone does not maintain conditions needed for efflux of the substrate  
216 EB. The addition of NaCl to the deionised water to yield a final concentration of 0.95% and a pH  
217 of 5.5 produced accumulation and efflux data similar to that of PBS of pH 5 and no glucose (data  
218 not shown).

219  
220 The ionophore CCCP is used for the demonstration of a PMF dependent efflux system.  
221 Interestingly, we have not been able to find a single study that shows where CCCP has its effects  
222 on the bacterial PMF. The brilliant papers by Mulkidjanian and colleagues (25-27) present strong

223 evidence that protons generated by metabolism are transported as hydronium ions via channels to  
224 the surface of the cell where they are distributed and bound to reactive groups of LPS. These  
225 hydronium ions are then transported via other channels (e.g. porins) to the periplasm of the  
226 Gram-negative bacterium where they are used by the pump of the efflux system for rapid  
227 extrusion of the substrate. KM Pos and colleagues recently published a series of studies and  
228 reviews demonstrating the path of the “proton” through the AcrB component of the efflux pump  
229 (28, 29).

230  
231 If we substitute the “proton” with the hydronium ion, the passage of the hydronium ion would  
232 establish the needed condition for the dissociation of the substrate from the transporter as  
233 predicted by Nikaido’s group (30). Because the latter group were able to show that the  
234 dissociation of the substrate is very slow at high pH (7 or greater) and very high at low pH (5.5),  
235 the passage of the hydronium ion would create the needed acid pH for dissociation of substrate  
236 from the transporter. Because CCCP would be expected to reduce the availability of hydronium  
237 ions to the periplasm, the substrate would remain tightly bound to the transporter and its  
238 extrusion would be slowed down or not at all. Employing varying CCCP concentrations in the  
239 deionised water efflux assay (pH 5.5), it would be possible to determine the effects on  
240 accumulation. As shown by Figure 6A the presence of CCCP at varying concentrations produces  
241 rapid and huge increases in the accumulation of EB when there is no glucose in the system. The  
242 amount of EB accumulated is significantly greater than that taking place in glucose-free  
243 deionised water. When glucose is present, the same concentrations of CCCP are less effective.  
244 The effect of the higher concentration of CCCP on efflux and the modulation of this effect by  
245 glucose is depicted by Figure 6B. It is evident that the inhibition of efflux by CCCP alone can be

246 eliminated by metabolic energy in deionised water of pH 5.5. These results suggest that a  
247 sufficient metabolic source of energy can overcome the effects of CCCP even when no ions are  
248 present in the system.

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250 **4. Discussion**

251 The activity of the main efflux pump of a Gram-negative bacterium has been previously shown to  
252 be modulated by pH and by metabolic energy (6, 9, 16, 17). At the low pH of 5, efflux activity is  
253 greater than at the high pH of 8. Whereas at high pH efflux is dependent upon metabolic energy,  
254 at low pH, efflux does not depend on metabolic energy, at least for the duration of the assays  
255 employed. In the current study, the efflux system of Salmonella Enteritidis NCTC 13349 is more  
256 effective at the low pH of 5 than at the high pH of 8; and at low pH it is less dependent of  
257 metabolic energy (glucose, ETOH, pyruvate). However, when ions are omitted from the medium,  
258 efflux is dependent upon metabolic energy even though the pH of the ion free milieu (deionised  
259 water) is low (pH 5.5). Sodium therefore appears to serve as an anti-port for efflux of the  
260 universal substrate EB. Or [Na] contributes to the chemical potential necessary for energising  
261 efflux.

262  
263 The phenothiazine thioridazine is known to promote accumulation of EB by inhibiting efflux of  
264 this substrate (19). In the current study, the inhibition of efflux by thioridazine has also been  
265 noted. However, the inhibition of efflux takes place at the high pH of 8 and not at the low  
266 pH of 5. Thioridazine is known to inhibit enzymes involved in metabolism (19). Because the  
267 presence of glucose does not reduce the inhibition of efflux produced by thioridazine, the  
268 inhibition of efflux at pH 8 is assumed to be the result of an inhibition of the enzyme system  
269 involved in metabolism.

270  
271 Summarising the results obtained in the current study, we can say that efflux is supported by ions  
272 and metabolic energy and that the degree of support provided by ions and energy is modulated by

273 pH. But how do these results relate to the mechanism by which AcrAB, the main efflux pump of  
274 Salmonella, functions? Crucial to understanding the mechanism of efflux lies in studies that  
275 demonstrate two main characteristics of the AcrAB transporter, namely, that the binding of the  
276 substrate (example EB) that takes place at neutral pH is stable (low dissociation constant), and,  
277 the dissociation of the substrate requires a pH lower than 6 (12, 28). Association and dissociation  
278 does not require metabolic energy as is the case for an ABC transporter (31, 32). Because  
279 dissociation of the substrate from the transporter must take place for the efflux pump to continue  
280 to extrude the noxious agent to the exterior of the cell, the internal pH of the transporter must be  
281 reduced to about 6 or lower for the dissociation of the substrate and its eventual release through  
282 the TolC conduit. It is the opinion of the authors that the reduction of internal pH of the  
283 transported takes place from the passage of hydronium ions that either come directly from  
284 metabolism when the pH of the milieu is high, or from the surface of the cell when the pH of the  
285 milieu is low (when the pH of the milieu is high, the dissociation of protons (hydronium ions)  
286 from the surface of the cell into the bulk medium is encouraged (25-27)). Results obtained in the  
287 current study suggest that the activity of the proton ionophore CCCP on efflux is modulated by  
288 pH. Because at high pH, CCCP has its greatest inhibition on the efflux of EB that is obviated by  
289 the availability of metabolic energy, we may assume that at high pH it is metabolism that  
290 provides the protons in the form of hydronium ions that as they pass through the internal cavity  
291 of the transporter, the pH is reduced to the level needed for the dissociation of the substrate. It is  
292 further suggested that the AcrAB-TolC provides the means by which molecules is extruded from  
293 the cell as a consequence of peristalsis provided by the movement of the AcrA fusion proteins as  
294 described by Eicher *et al.* (8).

295

296 When the pH of the milieu is low, the source of hydronium ions needed for passage through the  
297 transporter is assured due to the high concentration of surface bound protons (hydronium ions)  
298 (25-27). The difference between this high concentration of protons and that of the cytoplasm  
299 creates an electrochemical gradient (through the inner membrane) that manifests itself into the  
300 proton motive force (PMF). The movement of these protons is propagated by events within the  
301 periplasm and cytoplasm of the cell such as export functions. It is postulated that routinely, with  
302 export functions taking place, the need for hydronium ions is present and the translocation of  
303 these via the PMF, takes place through “aquaporins” (33). The result of the current study that  
304 shows that efflux at pH 5 does not require metabolic energy as long as ions are present in the  
305 system, is interpreted to reflect the availability of the required protons for passage through the  
306 transporter and subsequent reduction of pH resulting in the release of the substrate and  
307 subsequent extrusion provided by the flow of water to the surface of the cell. Lastly,  
308 extrapolation of the results of this current study to the in vivo passage of the Gram-negative  
309 bacterium that eventually colonises the colon, we may assume that during its passage through the  
310 stomach where the pH may be as low as 2, the bacterium is relatively immune to any noxious  
311 agent due to automatic efflux possible at this low pH. When it reaches the duodenum, the pH is  
312 higher but still quite acid (pH 4-5) and the bile salts present can easily be extruded automatically  
313 since the low pH of the environment will maintain a source of hydronium ions bound to the  
314 surface of cell, and hence, maintain the PMF required for movement of the hydronium ion to the  
315 periplasm and passage through the efflux pump thereby promoting release of the bile salt from  
316 the efflux pump and extrusion via the movement of water through the pump-possible assisted by  
317 the fusion protein promoted peristaltic movement. When the organism reaches the colon, the pH  
318 is near neutral. Here the organism faces toxicity presented by bile salts, which are recognised

319 when they are present in the periplasm and bound tightly to the transporter. If these toxins are to  
320 be released at the sites of the transporter where they are bound, the pH within the transporter  
321 must be reduced. It is here where metabolism of glucose and intermediates of metabolism furnish  
322 the needed hydronium ions for passage through the efflux pump, subsequent release of the  
323 substrate, and its expulsion into the medium.

324

325

326

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332 **Competing Interests:** None

333 **Ethical Approval:** Not applicable

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465 **Table 1. Minimum Inhibitory Concentration (MIC) of compounds used in this study.**

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Compound	MIC (mg/L)
	<i>Salmonella Enteritidis</i> NCTC 13349
EB	256 ug/ml
TZ	128 ug/ml
OV	> 5000 ug/ml
CCCP	25,6 ug/ml

468  
469 **EB**, ethidium bromide; **TZ**, thioridazine; **OV**, orthovanadate; **CCCP**, carbonyl cyanide m-  
470 chlorophenylhydrazone.

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504 **Title and Legends of Figures**

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506 **Figure 1A. Accumulation of EB at pH 5, 7.4 and 8 in absence of glucose**

507 **Legend.** Bacteria suspended in glucose free-saline buffered pH 5, 7.4 and 8 containing 1 mg/L

508 of EB incubated at 37C and accumulation of EB followed for 50 minutes. Note that at pH 5 no

509 accumulation of EB takes place from 1 through 50 minutes of assay. At pH 7.4 and 8

510 accumulation is essentially the same and increases for duration of assay.

511

512 **Figure 1B. Accumulation of EB at pH 5, 7.4 and 8 in presence of glucose**

513 **Legend.** Bacteria suspended in glucose-saline buffered pH 5, 7.4 and 8 containing 1 mg/L of EB

514 incubated at 37C and accumulation of EB followed for 50 minutes. Note that at pH 5 and 7.4 no

515 accumulation of EB takes place from 1 through 50 minutes of assay. At pH 8 EB begins to

516 accumulate after 30 or so minutes. Comparison of accumulation at pH 8 in absence (Figure 1A)

517 and presence of glucose (Figure 1B) indicates that the presence of metabolic energy reduces

518 significantly accumulation of EB.

519

520 **Figure 2. The effect of metabolic energy on efflux of EB at pH 8**

521 **Legend.** Accumulation of EB in glucose free-PBS pH 8 conducted for 30 minutes; the instrument

522 is paused and glucose free-PBS, PBS with glucose, pyruvate or ethanol is added; instrument re-

523 started and efflux of EB followed for an additional 30 minutes. Note that whereas accumulation

524 of EB continues with the addition of glucose free-PBS, addition of glucose promotes efflux.

525 Addition of Pyruvate or ethanol also promote efflux.

526

527 **Figure 3. The effect of CCCP on accumulation of EB at pH 5 and 8 in medium that**  
528 **contains glucose**

529 **Legend.** Accumulation of EB in glucose-saline pH 5 and 8 with and without 5 mg/L of CCCP.

530 **Note:** The effects of CCCP are concentration dependent with minimal effects with 2.5  
531 mg/L (data not shown).

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535 **Figure 4. The effect of TZ on the efflux of EB by salmonella in glucose saline pH 5 and 8**

536 **Legend.** Accumulation of EB at pH 5 and 8 in glucose-free saline; instrument paused and 10  
537 mg/L of TZ with glucose-saline of pH 5 and 8 added, and the instrument re-started.

538 **Note:** Effect of TZ on efflux is greatest at pH 8 than at pH 5.

539

540 **Figure 5A. Accumulation of EB by salmonella in deionised water pH 5.5 and modulation**  
541 **by glucose**

542

543 **Figure 5B. Efflux of EB by salmonella in deionised water pH 5.5 and modulation by glucose**

544

545 **Figure 6A. Effect of CCCP on accumulation of EB in deionised water pH 5.5**

546 **Note.** Presence of CCCP in glucose-free deionised water produces accumulation of EB in a  
547 concentration dependent manner. The amount of EB accumulated is significantly greater than that  
548 taking place in glucose-free water alone. The presence of glucose significantly reduces the  
549 amount of EB accumulated produced by CCCP. The glucose reduced level of CCCP accumulated  
550 is lower than that present in glucose-free deionised water.

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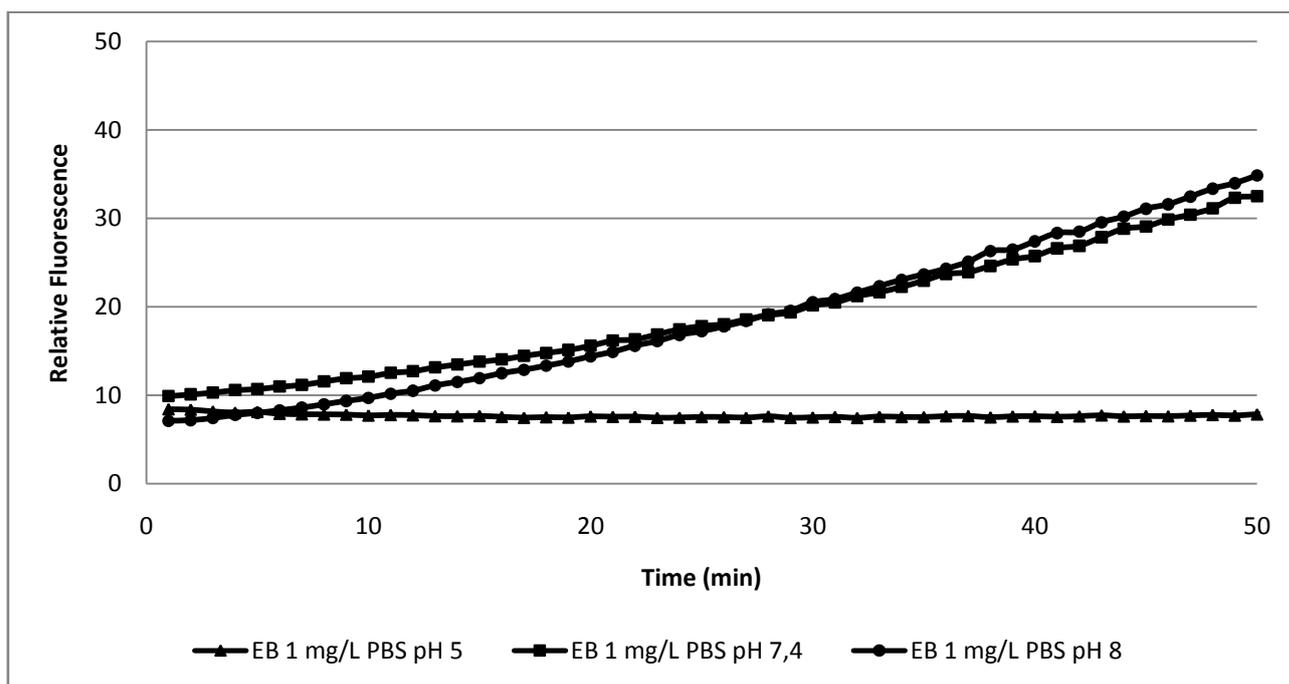
552 **Figure 6B. Effect of additions of CCCP on efflux of EB in deionised water pH 5.5**

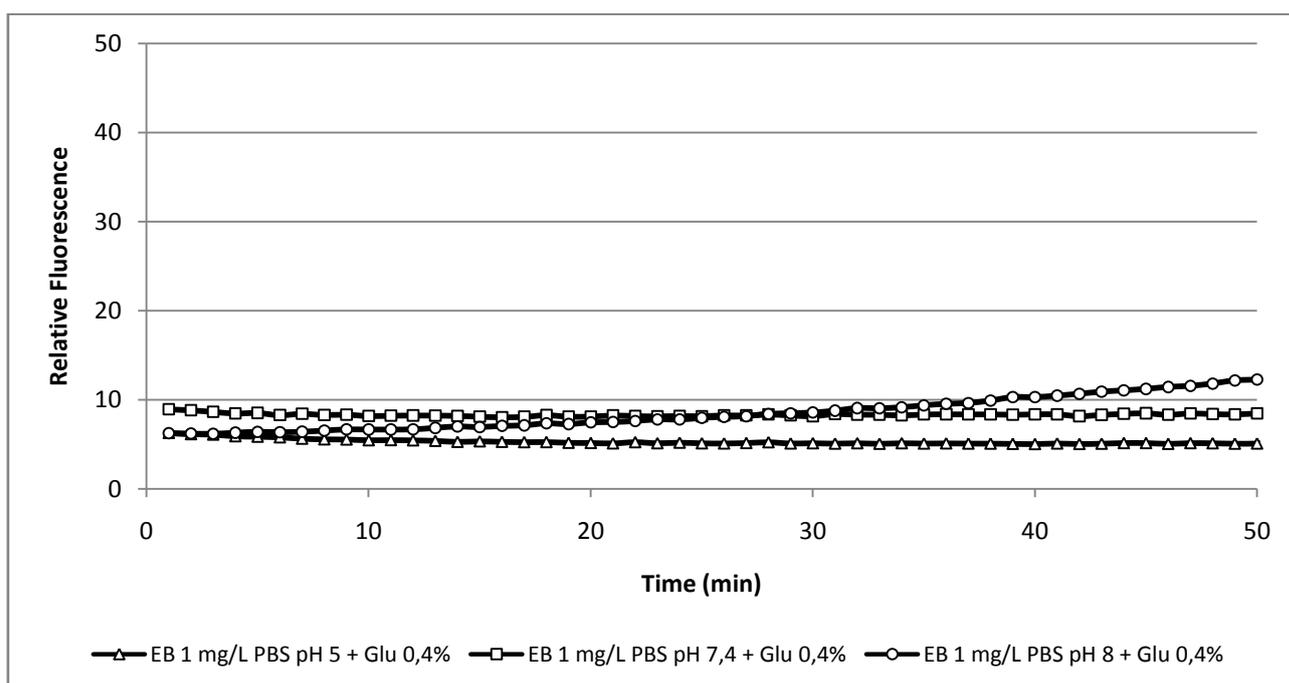
553 **Note.** Despite the addition of a high concentration of CCCP, efflux of EB takes place when the  
554 addition contains glucose.

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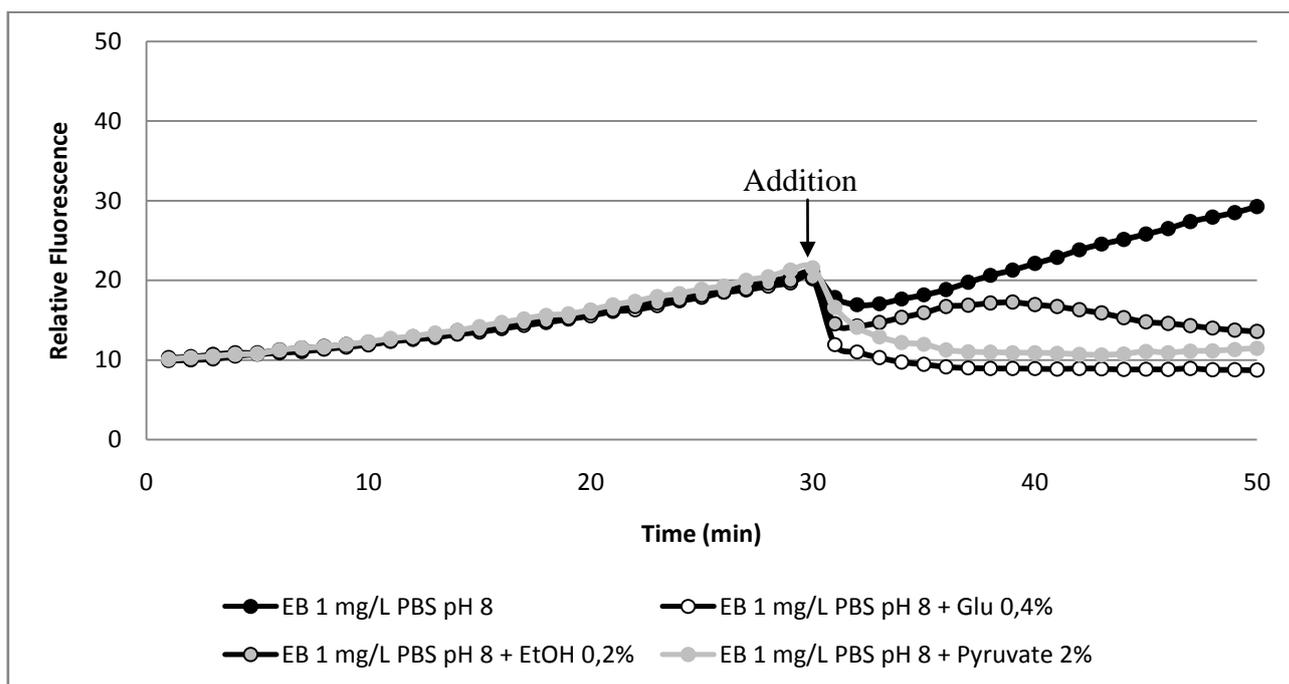
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**Figure 1A.** Accumulation of EB at pH 5, 7.4 and 8 in absence of glucose.

**Figure 1B.** Accumulation of EB at pH 5, 7.4 and 8 in presence of glucose.

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**Figure 2. The effect of metabolic energy on efflux of EB at pH 8.**

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**Figure 3A.** The effect of CCCP on accumulation of EB at pH 5 and 8 in medium that contain glucose.

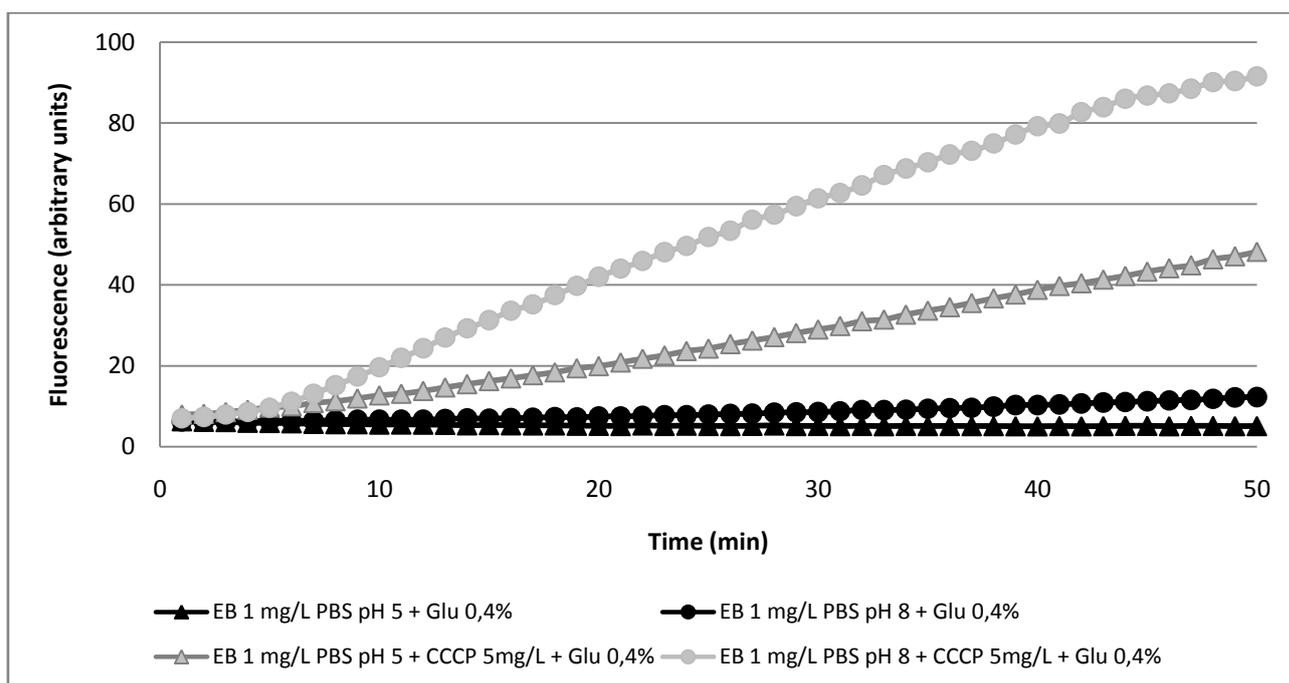
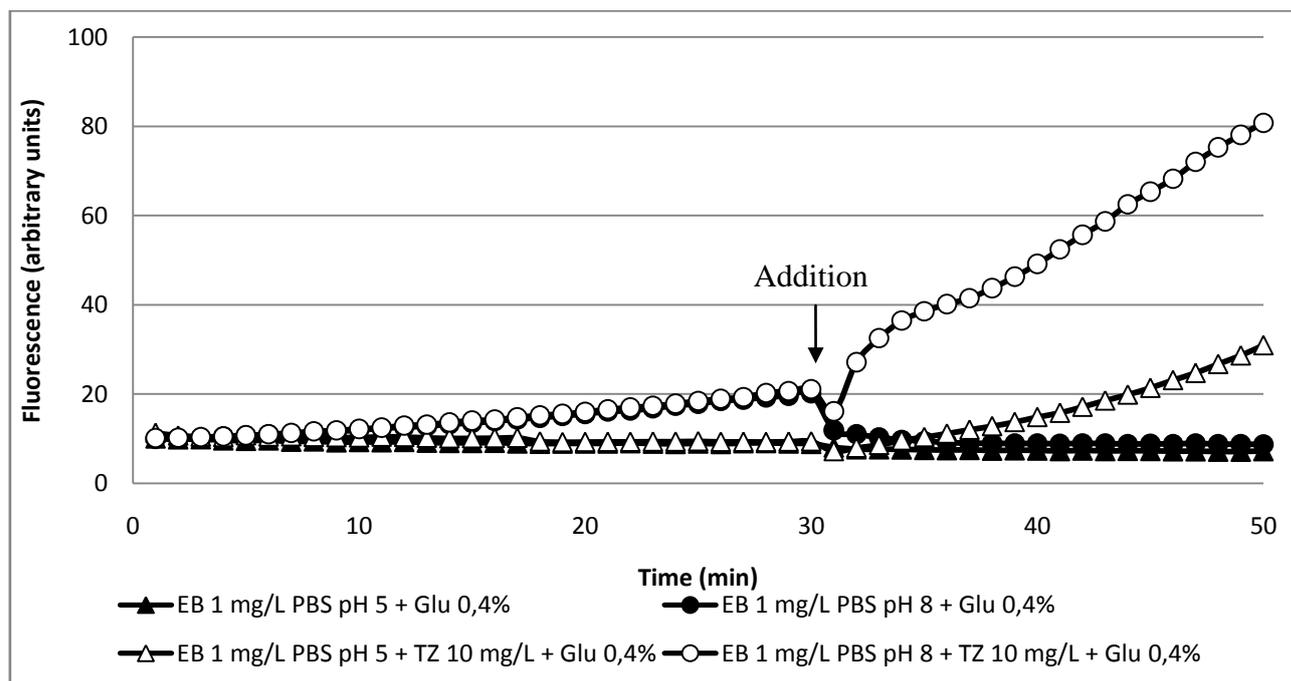
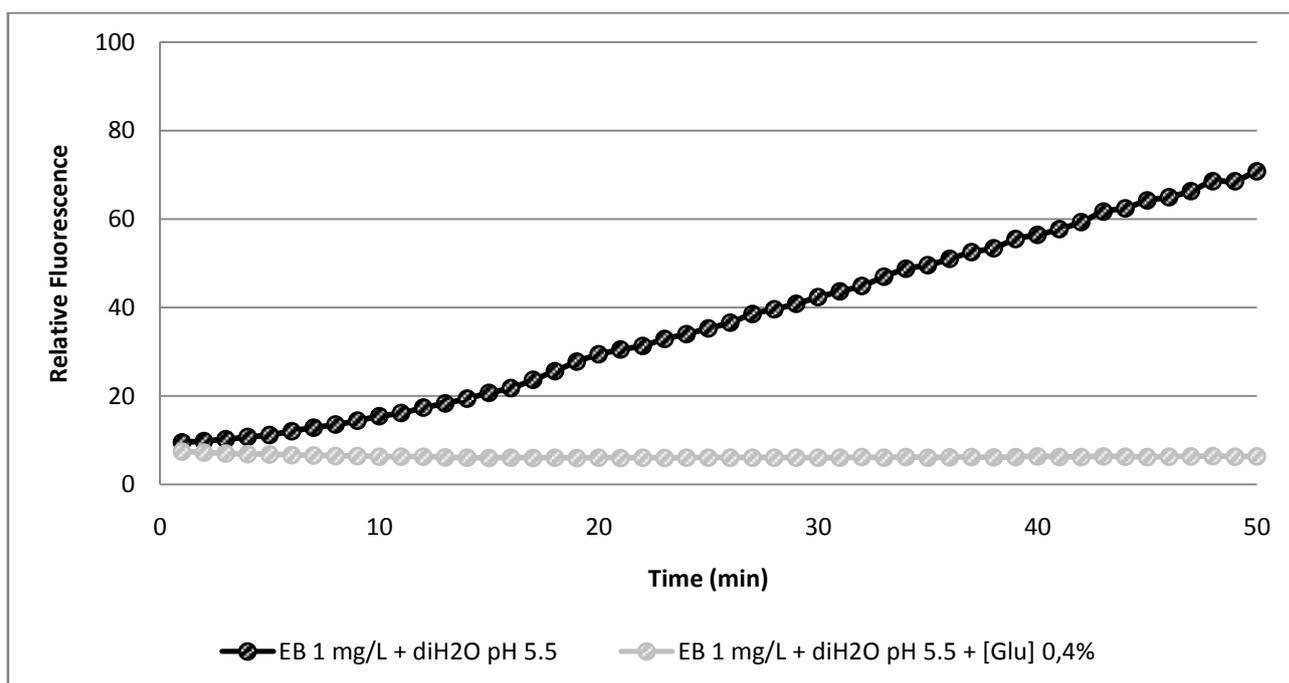


Figure 4. The effect of TZ on the efflux of EB by salmonella in glucose saline pH 5 and 8.

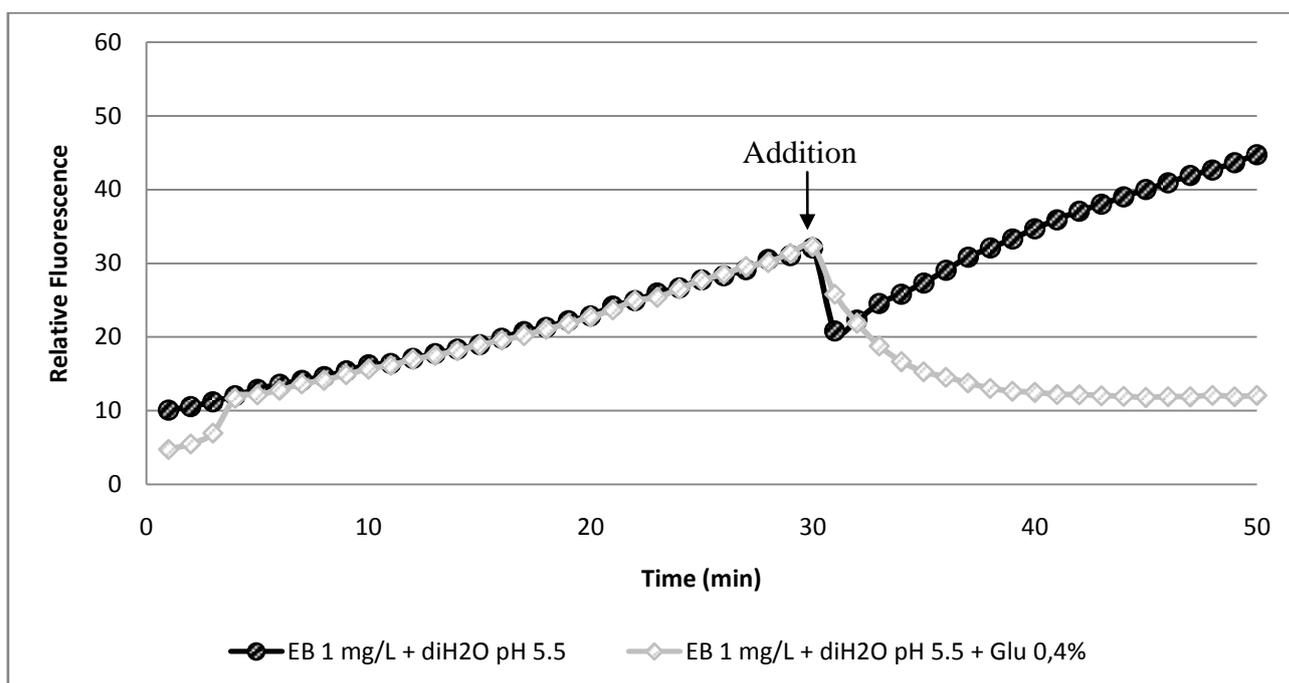


**Figure 5A. Accumulation of EB by salmonella in deionised water pH 5.5 and modulation by glucose.**



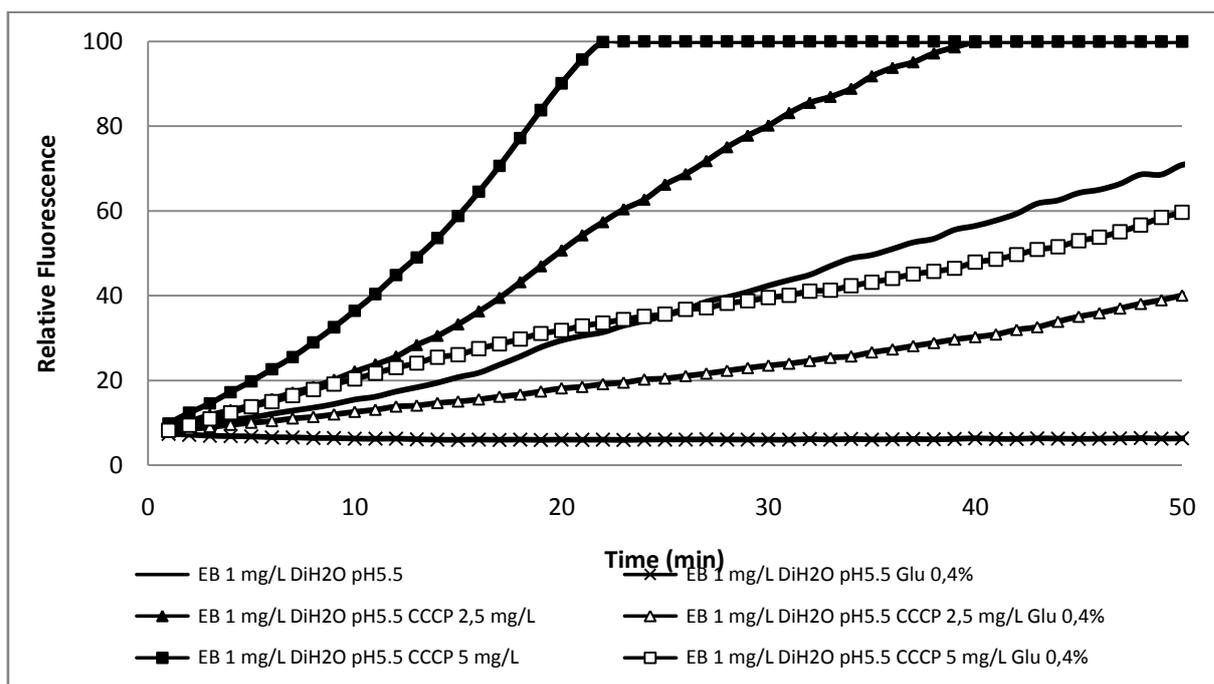
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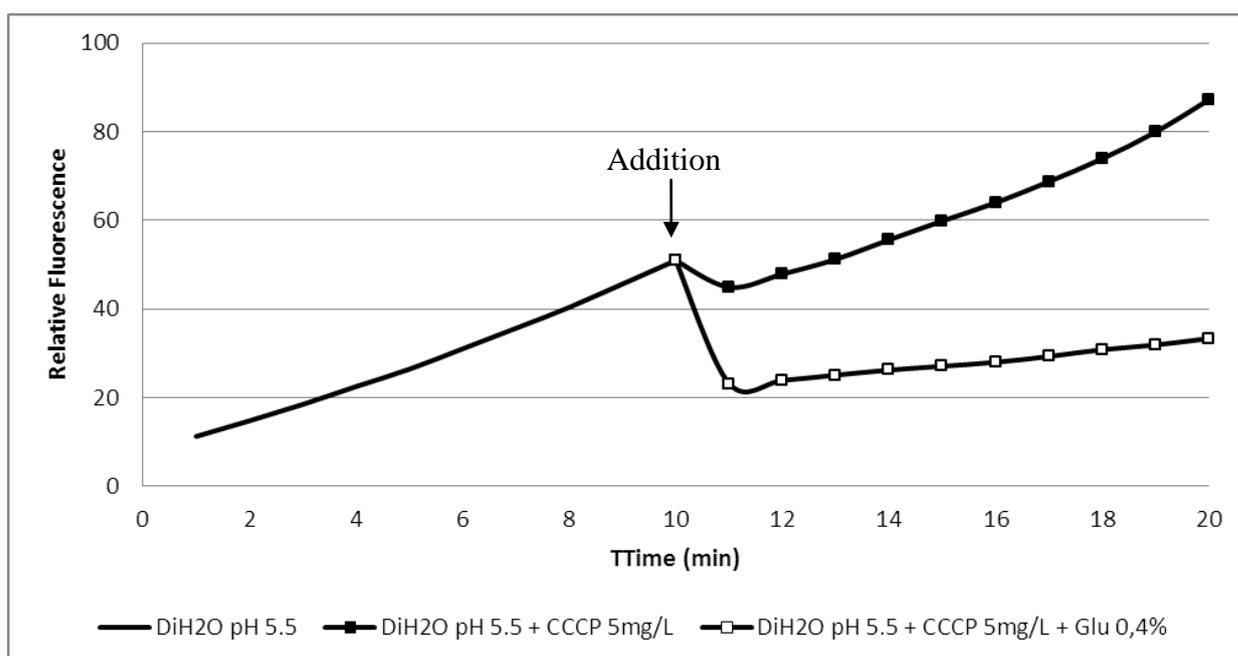
**Figure 5B.** Efflux of EB by salmonella in deionised water pH 5.5 and modulation by glucose.



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Figure 6A. Effect of CCCP on accumulation of EB in deionised water pH 5.5.



**Figure 6B.** Effect of additions of CCCP on efflux of EB in deionised water pH 5.5.

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