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8   The effect of interactions between a bacterial strain isolated from drinking water

9   and a pathogen surrogate on biofilms formation diverged under static versus flow

10   conditions

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21   Running headline: Interactions on biofilm formation

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26   Abstract

27   Aims

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28   Interactions with water bacteria affect the incorporation of pathogens into biofilms and

29   thus pathogen control in drinking water systems. This study was to examine the impact of

30   static versus flow conditions on interactions between a pathogen and a water bacterium

31   on pathogen biofilm formation under laboratory settings.

32   **Methods and Results**

33   A pathogen surrogate *Escherichia coli* and a drinking water isolate *Stenotrophomonas*

34   *maltophilia* was selected for this study. Biofilm growth was examined under two distinct

35   conditions, in flow cells with continuous medium supply versus in static microtiter plates

36   with batch culture. *E. coli* biofilm was greatly stimulated (~2-1000× faster) with the

37   presence of *S. maltophilia* in flow cells, but surprisingly inhibited (~65-95% less

38   biomass) in microtiter plates. These divergent effects were explained through various

39   aspects including surface attachment, cellular growth, extracellular signals, and

40   autoaggregation.

41   **Conclusions**

42   Interactions with the same water bacterium resulted in different effects on *E. coli* biofilm

43   formation when culture conditions changed from static to flow.

44   **Significance and Impact of Study**

45   This study highlights the complexity of species interactions on biofilm formation and

46   suggests that environmental conditions such as the flow regime can be taken into

47   consideration for the management of microbial contamination in drinking water systems.

48

49   **Keywords**

50   Biofilms; Drinking water; *E. coli*; Microbial contamination; Environmental

51

52   **Introduction**

53   Biofilms are ubiquitous in drinking water distribution systems (DWDS) and premise

54   plumbing (PP) (Simoes et al. 2006; Wang et al. 2014). Many waterborne disease

55   outbreaks are linked to biofilm growth (Lau and Ashbolt 2009). Incorporation in biofilms

56   can protect bacterial pathogens, which can be introduced through infiltration or

57   contamination, from residual disinfectants and other harsh stresses (Wingender and

58 Flemming 2011; Schwering et al. 2013; Burmolle et al. 2014; Sanchez-Vizuete et al.
59 2015). As a result, pathogens often survive and even proliferate in DWDS and PP
60 (Szewzyk et al. 2000; Donohue et al. 2015). A critical factor that determines whether a
61 pathogen can be incorporated in a biofilm is its interactions with persisting bacteria in
62 water systems. Synergistic interactions promote its biofilm formation, while antagonistic
63 interactions limit its embedding and growth in biofilms (Elias and Banin 2012; Burmolle
64 et al. 2014; Rendueles and Ghigo 2015). Whether an interaction is synergistic, neutral, or
65 antagonistic depends on the interacting water bacteria and environmental conditions
66 (Simoes et al. 2007; Klayman et al. 2009b). Environmental factors especially
67 hydrodynamics, and carbon/nutrient levels can modify mixed species biofilm formation
68 (Stoodley et al. 1998; Manuel et al. 2007; Zhang et al. 2013; Shen et al. 2015). The
69 modification by hydrodynamics for a complex, multiple-species community can be
70 achieved by selecting for certain bacteria against others, as microbial composition in
71 water biofilms change with flow condition (Douterelo et al. 2016). Within a dual-species
72 community, the modification may work by changing interactions between the two, as
73 suggested by one study showing impact of flow gradients on dual-species biofilm
74 formation (Zhang et al. 2013). However, it is unclear to what degree the interactions can
75 change from flow to static conditions and whether this change will result in significant
76 differences in pathogen survival. Although mixed-species interactions on biofilm
77 formation have been widely investigated, surprisingly very few studies have compared
78 this contrasting environmental condition, flow versus static, when evaluating dual-species
79 interactions.

80 Contrasting flow conditions are relevant to pathogen control in DWDS. Various sections
81 of DWDS, such as storage tanks and main pipes, differ greatly in water flow and
82 corresponding nutrient replenishment. Surveillance of drinking water-related outbreaks
83 revealed some cases of contamination originating from storage tanks (Kramer et al. 1996)
84 and others from DWDS and PP pipes (Brunkard et al. 2011; Beer et al. 2015). One study
85 particularly found that higher occurrence of coliforms was associated with DWDS
86 containing more water tanks (LeChevallier et al. 1996). These reports suggested
87 differential pathogen survival in pipes and water tanks. How contrasting flow conditions

88 may contribute to such differences deserves systematic investigation in well controlled
89 and replicable laboratory settings.

90 Laboratory studies to evaluate the interactions between bacterial isolates are most often
91 conducted either using static conditions, namely microtiter plates as the most commonly
92 used apparatus, or in continuous flow systems such as flow cells or flow chambers
93 (Stoodley et al. 1998; Zhang et al. 2013; Burmolle et al. 2014). Very few studies use both
94 conditions to investigate interactions between the same bacteria. We hypothesized that
95 interactions between the same two bacteria and the resulting impact on biofilm growth
96 can change from static to flow conditions. Similar observations were reported for single
97 species or complex multiple-species biofilm formation in flow/no-flow conditions
98 (Mampel et al. 2006; Manuel et al. 2007). We aimed to test this hypothesis, by using a
99 dual species model consisting of a bacterial strain isolated from drinking water and a
100 pathogen surrogate and culturing the two bacteria in static microtiter plates and
101 continuous flow cells.

102 *Escherichia coli* is an indicator bacterium of fecal contamination in water resources. Its
103 detection suggests the presence of pathogens originating from fecal contamination. Thus,
104 it was used as a surrogate for pathogenic species. *Stenotrophomonas maltophilia* is
105 frequently detected among heterotrophic plate count isolates from DWDS and PP water
106 and biofilms (Critchley et al. 2003; Simões et al. 2007), and especially in hospital potable
107 water (Safdar and Rolston 2007). The relative abundance of *Stenotrophomonas* spp. was
108 reported to be 1-6% among isolates from various sampling sites in a pilot-scale DWDS
109 (Norton and LeChevallier 2000). *S. maltophilia* is the third most common isolates
110 (13.5%) from treated, tap, and haemodialysis effluent water (Arvanitidou et al. 2003).
111 The number of *S. maltophilia* reached 49 CFU ml^{-1} in water reservoirs of dental clinics
112 (Szymańska 2007) and was recently detected at relatively high levels in biofilms
113 collected from faucet aerators in 15 homes using qPCR targeting the 23S rRNA gene
114 (Haig et al. 2016). It is also of clinical relevance as one of the most frequently isolated
115 opportunistic pathogens among cystic fibrosis patients (Waters et al. 2011). *S.*
116 *maltophilia* was thus used here to represent a persisting water bacterium. Because both *E.*
117 *coli* and *S. maltophilia* have been isolated together from water systems and other

118   environments where biofilms are important (Arvanitidou et al. 2003; Rudi et al. 2009), a

119   scenario of their co-existence and interactions is plausible and relevant.

120   We aimed to evaluate the impact of contrasting flow/static conditions on dual-species

121   interactions with regard to *E. coli* biofilm formation under laboratory settings. We studied

122   how a drinking water bacterium *S. maltophilia* affected biofilm formation of a pathogen

123   surrogate *E. coli* in flow cells and static plates, representing different flow conditions in

124   drinking water systems. We further explored several aspects of biofilm formation of *E.*

125   *coli,* including cell growth, surface attachment, diffusible signals, and cell aggregation, in

126   order to understand the observed difference in species interactions due to culture

127   conditions.

128   Materials and methods

129   Bacterial strains and cultures

130   *E. coli* K-12 PHL644 and *S. maltophilia* were used in this study. The *E. coli* strain is a

131   good biofilm-former due to a mutation in gene *ompR* and thus exhibits an increase in

132   curli expression (Vidal et al. 1998). It was chosen to simulate a worse-case scenario in

133   pathogen control where the incoming pathogens are efficient in biofilm formation by

134   themselves. The *S. maltophilia* strain was isolated from drinking water and identified by

135   sequencing its full length 16S rRNA gene. Both strains were tested to be sensitive to

136   tetracycline and gentamicin. To facilitate the differentiation of the two strains, plasmids

137   pMP4655-GFP and pBPF-mCherry were transformed into *E. coli* and *S. maltophilia,*

138   respectively, by electroporation using a Gene Pulser Electroporation System (Bio-Rad,

139   Hercules, CA) following the manufacture's protocol. Transformed *E. coli* and *S.*

140   *maltophilia* were selected by culturing onto Luria-Bertani (LB) agar with 40 μg ml^{-1}

141   tetracycline or with 20 μg ml^{-1} gentamicin (Sigma-Aldrich, St. Louis, MO)*,* respectively.

142   The constitutively expressed green fluorescence protein (GFP) in *E. coli* and red

143   fluorescence protein (mCherry) in *S. maltophilia* were both confirmed with fluorescence

144   microscopy. Growth rate and biofilm formation of transformed strains were not different

145   from the ones without a plasmid (data not shown). Strains were stored in LB broth with

146   20% glycerol in -80°C. For active culture, strains were streaked from glycerol stocks onto

147 LB agar with antibiotics (40 μg ml^{-1} tetracycline for *E. coli* or 20 μg ml^{-1} gentamicin for

148 *S. maltophilia*). Single colonies from agar plates were used to inoculate broth cultures.

149 Broth medium was 10-fold diluted LB broth (0.1×LB). This diluted broth was chosen to

150 approximate oligotrophic drinking water but to still have higher levels of essential

151 nutrients than typical drinking water to allow biofilms to grow and mature within days for

152 laboratory study (Table S1). Other media such as undiluted Reasoner's 2A (R2A) or

153 diluted Trypticase Soy Broth (TSB) with similar nutrient levels and ionic strength as in

154 our 0.1×LB have been used to study biofilm formation of drinking water related bacteria

155 (Table S1) (Simoes et al. 2007; Klayman et al. 2009b; Simoes et al. 2010). Antibiotics

156 were supplemented into 0.1×LB to maintain plasmids. Broth cultures were incubated at

157 30 °C overnight (13 h) with vigorous shaking (250 rpm). To wash off any residual

158 antibiotics, cells of *E. coli* or *S. maltophilia* were pelleted by centrifugation (3,000×g, 3

159 min) and re-suspended in antibiotic-free fresh 0.1×LB for inoculation into biofilm

160 systems. Cell densities of *E. coli* and *S. maltophilia* in inoculum were quantified by plate

161 counting.

162 **Biofilm cultures in flow cells**

163 Flow systems were assembled with three-channel glass-bottom flow cells (Stovall,

164 Greensboro, NC), each channel with dimensions of 1×4×40 mm. Antibiotic-free 0.1×LB

165 broth was supplied at 0.12 ml min^{-1}, resulting a laminar flow (Reynolds number =0.8)

166 and low flow velocity (=0.5 mm s^{-1}) which is in the low range of flows in DWDS. The

167 medium flow was paused for inoculation. One milliliter cell inoculum of *E. coli*, *S.*

168 *maltophilia* or a mixture of the two was injected into each flow cell, and allowed to attach

169 onto surfaces for one hour. Five flow cells were run in parallel (E1, S, E1mix, E0.1,

170 E0.1mix). Labels E1 and E0.1 represent a 10-fold difference in the inoculum of *E. coli*

171 (10^9 CFU ml^{-1} and 10^8 CFU ml^{-1}, respectively). Inoculated *S. maltophilia* was 10^9 CFU

172 ml^{-1} in both mixed cultures (E1mix, E0.1mix) and the pure culture control (S). Medium

173 flow was resumed and this time point was recorded as 0 h. Growth of biofilms was

174 monitored with confocal laser scanning microscopy (details in the section of 'Imaging

175 biofilms') at various time points until mature biofilms developed without observable

176 change in biomass or structure or 96 h. The flow cell system was operated at 20 °C and

177 replicated independently.

178 **Biofilm cultures in static plates**

179 Mono- (E1, E0.1, S) and mixed-species (E1mix, E1mix[#], E0.1mix) biofilms were grown

180 in 96-well Nunclon microtiter plates (Fisher Scientific, Pittsburgh, PA) and in 24-well

181 glass-bottom plates (MatTek, Ashland, MA). Re-suspended cells from overnight

182 planktonic cultures were washed and inoculated into 0.1×LB broth (100 μl per well) with

183 no antibiotics supplemented in either pure or mixed cultures. Labels E1 and E0.1

184 represent an inoculum of 1 μl 10^9 CFU ml^{-1} and 10^8 CFU ml^{-1} per 100 μl medium,

185 respectively. Inoculated *S. maltophilia* was 1 μl 10^9 CFU ml^{-1} per 100 μl medium in

186 E1mix, E0.1mix and S, but 10-fold less (1 μl 10^8 CFU ml^{-1}) in E1mix[#]. The plates were

187 left static for biofilm growth at 20 °C for 22 h. Planktonic cells in each well were gently

188 removed and washed three times with phosphate buffered saline (PBS, pH 7.2). Biofilm

189 growth in 96-well plate was quantified using a crystal violet (CV) staining method

190 (O'Toole and Kolter 1998) and biomass was shown as OD600 (optical density at 600 nm)

191 in arbitrary units. Four replicate cultures were grown for each type of biofilm in the same

192 plate. Biofilm in 24-well plate was imaged with confocal laser scanning microscopy

193 (details in the section of 'Imaging biofilms'). Biofilm cultures were replicated three times

194 independently.

195 **Test the effect of diffusible signals**

196 Planktonic cultures of *S. maltophilia* were harvested at 4 h (exponential phase) and 15 h

197 (stationary phase) after inoculation into 0.1×LB broth. No antibiotics were supplemented

198 in these cultures. Supernatant was acquired by filtering planktonic cultures through

199 membrane filters (0.22 μm, Millipore, Billerica, MA). Culture medium (0.2×LB broth)

200 was supplemented with equal volume of the supernatant for biofilm growth in 96-well

201 microtiter plates.

202 To test the impact of diffusible signals, biofilm cultures were also conducted in Transwell

203 systems (Corning, NY). Each Transwell consists of a 24-well microtiter plate and 24

204 inserts, one per well. The insert has a polycarbonate membrane (0.4 μm) bottom to

205   separate bacterial cells grown in the insert from those grown in the microtiter well, but

206   allowing the culture medium and diffusible signals being exchanged between each pair of

207   insert and well during the period of incubation. *E. coli* was inoculated into the microtiter

208   wells (1 μl 10^9 CFU ml^{-1} per 100 μl medium), while *S. maltophilia* or a mixture of *E.* coli

209   with *S. maltophilia* (equal number pool) was inoculated into the inserts (1 μl 10^9 CFU ml^{-}

210   ^{1} per 100 μl medium). After 22-h incubation in static at 20 °C, the inserts were discarded

211   and *E. coli* biofilms grown in the 24-well microtiter plates were quantified with the

212   method of CV staining.

213   **Initial attachment assay**

214   Overnight cultures of *E. coli* and *S. maltophilia* were re-suspended in fresh 0.1×LB (no

215   antibiotics) and adjusted to be 10^9 CFU ml^{-1}. Pure *E. coli, S. maltophilia* or their 1:1

216   mixtures were added into a 96-well plate (100 μl per well) and left static at 20 °C for the

217   1-h initial attachment. Suspended cells were gently removed and washed three times with

218   PBS. Biomass of attached cells was quantified with the method of CV staining.

219   **Cell autoaggregation**

220   Overnight cultures of *E. coli* and *S. maltophilia* were resuspended in PBS buffer. *E. coli*

221   suspension, or mixed suspension of *E. coli* with 10-fold less or the same amount of *S.*

222   *maltophilia* cells were prepared. Three mililiter of these suspensions were added into a 5-

223   ml test tube (Fisher Scientific, Pittsburgh, PA) and set static for 24 h. Colony forming

224   unit of *E. coli* in the topmost suspension column (sampled at 0 h and 24 h after setting) in

225   the test tubes were measured by plate counting with no sample homogenization. The

226   reduction of CFU density reflected the degree of *E. coli* cell aggregation. The

227   experiments were repeated independently twice.

228   **Biofilm invasion**

229   Pure *E. coli* or *S. maltophilia* were allowed for an 1-h initial attachment in microtiter

230   plates the same way as described above. After the 1-h initial attachment, suspended cells

231   of this species were either gently removed or kept in the well. Suspension of the other

232   species was added into the wells to invade the pre-attached species. The invasion lasted

233   for one hour. Attached biomass was then washed and quantified with the CV staining

234   method. A similar invasion experiment was also performed using well-developed (24-h

235   growth after the inoculation) rather than the 1-h pre-attached biofilms in glass-bottom

236   plates (MatTek, Ashland, MA). Biofilms before and after invasion were imaged with

237   fluorescence microscopy (Olympus, Wirtz, VA). The invasion experiments were repeated

238   three times with similar results.

239   **Imaging biofilms**

240   Images of biofilms were acquired with a confocal laser scanning microscope

241   (Fluoview[TM], Olympus, Wirtz, VA) with filter sets for monitoring GFP and mCherry

242   fluorescence in *E. coli* and *S. maltophilia*, respectively. Images were obtained randomly

243   from three to six spots in the center of each flow chamber or each well of the microtiter

244   plates. Biofilms grown near the edge of a flow chamber were acquired only if no cells

245   were observed in the center of a flow chamber in the pure *E. coli* culture with low

246   inoculation (system E0.1). Three-dimensional images were reconstructed using the

247   software Volocity 3.2 (Improvision Inc., Waltham, MA) from a stack of confocal

248   microscopy images for the x-y sections of biofilm samples. Stacks of confocal images

249   were also analyzed for biomass quantification following the manual of COMSTAT

250   (Heydorn et al. 2000).

251   **Motility test and statistics**

252   The swimming and swarming ability of *E. coli* and *S. maltophilia* was tested on soft agar

253   LB plates (0.3% agar for swimming and 0.5% for swarming) similar to previously

254   described protocol (Deziel et al. 2001). Student t tests were performed to test whether the

255   difference between two groups was significant ($p < 0.05$) or not.

256   **Results**

257   **The presence of *S. maltophilia* stimulated *E. coli* biofilm growth in flow cells**

258   To investigate how *E. coli* biofilm growth was affected by *S. maltophilia*, we compared

259   two mixed-species cultures (E1mix and E0.1mix) in flow cells with two mono-species *E.*

260   *coli* cultures as controls (E1 and E0.1) (Figure 1). Biofilms were imaged at various time

261   points. Three-dimensional images were constructed showing both strains (Figure S1) or

262   showing only *E. coli* cells (Figure 1A) to facilitate the comparison of *E. coli* biofilm

263   formation among different cultures. Biomass of *E. coli* or *S. maltophilia* was quantified

264   based on microscopy images (Figures 1B and S2).

265   Cells of *E. coli* initially attached onto surfaces in all flow cells during the one hour of

266   inoculation before flow was started. However, most attached cells in mono-species

267   systems were quickly (<5 h in E0.1) or gradually (<33 h in E1) washed away once flow

268   resumed. More than 99% and 85% of *E. coli* biomass was detached from biofilms in a 7-

269   h time period (from 1 h to 8 h after starting the flow) in pure cultures E0.1 and E1,

270   respectively. *E. coli* biomass further decreased and reached a lowest value at 33 h after

271   starting the flow in system E1 (biomass 0.09 $\mu m^3/\mu m^2$). Afterwards, biomass increased

272   slightly until microcolonies grew (55 h after starting the flow) and spread until a blanket

273   of biofilm formed 80 h after starting the flow (biomass 5.47 $\mu m^3/\mu m^2$). In system E0.1,

274   very few *E. coli* cells were observed in the flow cells between 55 h and 96 h after starting

275   the flow. Some loosely attached cells were observed near the edge of the flow chamber,

276   where shear force is close to zero (biomass < 0.04 $\mu m^3/\mu m^2$). Massive detachment of

277   initially attached cells caused by the flow seems to be the major obstacle of *E. coli*

278   biofilm development.

279   In contrast, detachment of *E. coli* was transient and much less severe in mixed-species

280   flow cells. In E1mix, less than 10% of *E. coli* biomass was lost in a 7-h time period (from

281   1 h to 8 h after starting the flow). *E. coli* biomass steadily increased and reached a similar

282   level (5.59 $\mu m^3/\mu m^2$) as in mono-species culture E1 (5.47 $\mu m^3/\mu m^2$ at 80 h) in

283   approximately half the time (45 h, ~2× faster). The stimulation was more obvious when

284   10-fold less *E. coli* cells were inoculated (E0.1mix vs E0.1). The biomass of biofilms

285   increased steadily from the first time point and reached a biomass level of 4.89 $\mu m^3/\mu m^2$

286   at 45 h in E0.1mix, in comparison to the no observable biofilms (<0.01 $\mu m^3/\mu m^2$) in E0.1

287   by 96 h (~1000× faster). Based on these observations, the transient and greatly reduced

288   detachment of initially attached *E. coli* in the presence of *S. maltophilia* contributed to its

289   expedited biofilm formation in mixed culture in flow cells.

290   A mono-species *S. maltophilia* flow cell (S) was run as another control. A single layer of

291   cells was initially attached. A steady increase of biomass was observed (Figure S2).

292   Biomass quantification of *S. maltophilia* based on confocal microscopic images was not
293   performed beyond 17 h after starting the flow, as accurate quantification of biomass was
294   impossible since the fluorescent protein mCherry faded severely after 17 h. The
295   fluorescence fading was recognized when comparing fluorescent images with white light
296   images of biofilms within the same scope field of view, an example of which was shown
297   in Figure S3. The growth of *S. maltophilia* in mixed-species biofilms was similar to its
298   growth in mono-species culture within the first 17 h when accurate biomass
299   quantification was available (Figure S2). No loss of initially attached *S. maltophilia* was
300   observed at least within the first 17 hours, indicating its robust surface attachment. This
301   solid attachment seemed to help retain *E. coli* cells on the surface, which resulted in
302   stimulated *E. coli* biofilm growth described above.

303   **The presence of *S. maltophilia* inhibited *E. coli* biofilm formation in static plates**

304   Plastic microtiter plates were inoculated and incubated statically for biofilm growth in
305   mono-species (named as E1, E0.1, and S) and mixed-species cultures (named as E1mix,
306   E1mix[#], E0.1mix). The biomass of biofilms (mixed- or mono-species) was quantified
307   after 22 hour of incubation. The mono-species *E. coli* biofilm had the highest biomass
308   level of 2.73 (arbitrary unit as optical density at 600 nm) in E1, a slightly lower biomass
309   in E0.1 (2.07), while pure *S. maltophilia* biofilm had a biomass level of only 0.17 (94%
310   less than E1, 92% less than E0.1, $p<0.01$) (Figure 2A). All three mixed-species biofilms
311   had significantly less biomass than pure *E. coli* cultures (E1 or E0.1) (biomass=0.15-0.95,
312   ~65-95% less, $p<0.001$), regardless of the inoculum ratios of *E. coli* and *S. maltophilia*
313   (1:1 in E1mix, 0.1:1 in E0.1mix, and 1:0.1 in E1mix[#]). These results suggest a significant
314   inhibition of *E. coli* biofilm formation in mixed culture, although the contribution of each
315   species to the total biomass of mixed-species biofilms could not be determined with the
316   CV staining method.

317   The observed inhibition to *E. coli* biofilm formation was independent of the surface
318   materials when running with 0.1×LB broth, as a similar inhibition was observed in glass
319   microtiter plates (Figure 2). We examined the species composition of these dual-species
320   biofilms using microscopy images. Pure *E. coli* formed a multi-layer biofilm (E1), while
321   *S. maltophilia* (S) barely formed a single layer of cells (Figure 2B). Mixed-species

322 biofilms (E1mix, E1mix[#]) contained a single layer of *S. maltophilia* interspersed with

323 microcolonies of *E. coli*, whose biomass was much less than that in E1 (quantified as ~10%

324 for E1mix, Figure 2C) . The images confirmed the inhibition on *E. coli* biofilm formation

325 when co-cultured with *S. maltophilia*.

326 *E. coli* exhibited less planktonic cell growth in mixed culture than in pure culture

327 To identify whether the divergent effects in the flow cells versus the static cultures were

328 due to cell growth differences rather than to differences related to biofilm growth, we

329 measured growth rates and yields of the two species in planktonic cultures. *E. coli* had a

330 slightly lower maximum growth rate (generation time 67±5 min) than *S. maltophilia*

331 (generation time 59±2 min, p>0.05). We then measured yields of *E. coli* by quantifying

332 its cell numbers in planktonic cultures with the same inoculum under the same conditions

333 used for the biofilm cultures in microtiter plates. The number of *E. coli* cells in mixed

334 cultures (E1mix and E1mix[#]) was 18-33% less than in *E. coli* pure culture (E1, p>0.05)

335 (Figure 3). The less growth of *E. coli* in mixed culture was more obvious when starting

336 from 10-fold less *E. coli* (66% less *E. coli* in E0.1mix compared to in E0.1, p<0.05).

337 Diffusible signals of *S. maltophilia* impacted *E. coli* biofilm formation

338 To explore whether diffusible signals of *S. maltophilia* played a role in the observed

339 divergent effects in the two systems, we harvested the supernatants of *S. maltophilia* from

340 an exponational and a stationary growth phase and supplied them into the growth

341 medium (1:1 mixed with 0.2×LB broth to make it comparable with the 0.1×LB broth) for

342 culturing *E. coli* biofilms in microtiter plates. The biomass of *E. coli* biofilms was no

343 different to the no supernatant control (Figure S4). Considering that extracellular signals

344 may have a short shelf-life after being produced, thus may be missed from the

345 supernatant harvesting at the two predetermined timepoints, we used the Transwell

346 systems (Corning, NY) to test the impact of signals produced and diffused anytime

347 during the growth phases. These Transwell systems allowed the separation of a pure *E.*

348 *coli* biofilm growth in a microtiter well from the growth of *S. maltophilia* cells or a

349 mixed-culture in the insert of that well by a 0.4 μm membrane. Signals smaller than 0.4

350 μm should be allowed to diffuse from the insert into the well. We observed a 14%-21%

351 decrease in *E. coli* biofilm formation in the bottom well when *S. maltophilia* or mixed-
352 species were grown in the insert (p<0.05, Figure 4). Since nutrients and organic carbon
353 can also freely diffuse between the inserts and wells, we cannot completely exclude the
354 potential impact of resource competition on *E. coli* biofilm formation in these Transwell
355 systems. Thus, diffusible signals from *S. maltophilia*, possibly combined with nutrient
356 competition can induce up to 20% of observed inhibition on *E. coli* biofilm growth in
357 static batch culture.

358 ### *E. coli* **exhibits weak surface attachment**

359 Different bacteria in mixed-species biofilms compete for limited surface area during
360 attachment. We explored how *S. maltophilia* affected *E. coli* during surface attachment
361 while excluding potential cofactors of cellular growth, nutrient competition and diffusible
362 signals by depositing the same amount of *E. coli* and *S. maltophilia* cells in fresh medium
363 into microtiter plates. The 1-h initial attachment (Figure 5) showed the same trend as the
364 longer-term (22 h) biofilm growth in microtiter plates (Figure 2A). After 1 h contact with
365 the surface, *E. coli* exhibited four-fold greater attached biomass than *S. maltophilia*
366 (p<0.05, Figure 5). However, when mixed with *S. maltophilia,* the overall attachment
367 dropped to 29% (p<0.05, Figure 5), suggesting that initial attachment of *E. coli* was
368 greatly reduced in the presence of *S. maltophilia* cells. These results were acquired when
369 the microtiter plate was kept static.

370 In flow cells, shear force due to continuous flow impacts surface attachment. We
371 introduced some shear force into the microtiter culture by very gently shaking the
372 microtiter plate (60 rpm). As a result, *E. coli* formed 70% less biofilms compared to that
373 in static culture, while *S. maltophilia* biofilm growth was barely affected (Figure S5). It
374 suggests that the attachment of *E. coli* cells to the solid surface was fairly weak, while *S.*
375 *maltophilia* showed the opposite, less in biomass but relatively strong in the attchment. It
376 corresponded well with the observation of the massive loss of attached *E. coli* cells but
377 not *S. maltophilia* in flow cells after the flow resumed (Figure 1).

378 We further performed a series of invasion experiments to examine whether one species
379 can outcompete the other in surface attachment. Cells of one species (invaded species)

380   were deposited in microtiter wells for the 1-h pre-attachment. With or without removing

381   planktonic cells of the invaded species, suspension of the other species (invading species)

382   were added into the wells to invade the pre-attached biofilm. About 92% of pre-attached

383   *E. coli* biomass was lost after the invasion by *S. maltophilia* cells (Figure 6). It was

384   reasonable to assume that *S. maltophilia* cells can "remove" the majority of pre-attached

385   *E. coli* cells. The presence of planktonic *E. coli* cells showed no effect on the invasion of

386   *S. maltophilia.* In contrast, the presence of planktonic *S. maltophilia* affected the invasion

387   of *E. coli* to the pre-attached *S. maltophilia.* Only when planktonic *S. maltophilia* cell

388   were removed, were the invasion of *E. coli* successful with an increased biofilm biomass,

389   which was 3.9× more (Figure 6). Similar results were observed when well-developed

390   (grown for 24 h) *E. coli* biofilms on glass surface were invaded by *S. maltophilia* cells,

391   resulting in great loss of attached *E. coli* cells and a replacement of a layer of *S.*

392   *maltophilia* cells (Figure S6). The microscopic images (Figure S6) confirmed the

393   assumption that attached *E. coli* can be "removed" by *S. maltophilia* cells, while in the

394   other way, *E. coli* cells barely succeeded in attching onto a surface to which a single layer

395   of *S. maltophilia* cells had occupied while with the presence of free-living *S. maltophilia*

396   cells around.

397   **Autoaggregation of *E. coli* reduced in the presence of *S. maltophilia* cells**

398   Cell autoaggregation is critical for biofilm growth. The *E. coli* strain used in this study

399   can autoaggregate due to a mutation in the *ompR* gene (Vidal et al. 1998). We examined

400   how the presence of *S. maltophilia* affected the aggregation of *E. coli* cells. In a static

401   suspension column, cells aggregated and may settle down by gravity, resulting a

402   decreased cell density in the top layer of the column, especially when cell density was

403   measured with the method of plate counting (one aggregate grows into one colony

404   forming unit, CFU). The *E. coli* cell density in the top layer showed three orders of

405   magnitude decrease in its CFU after being static for 24 h (Figure 7), while *S. maltophilia*

406   showed no decrease (data not shown). The decrease was alleviated to only one or two

407   orders of magnitute when *E. coli* was mixed with an equal number or a 10-fold less

408   numbers of *S. maltophilia* cells (Figure 7), respectively. It indicated that planktonic *S.*

409   *maltophilia* cells can reduce the autoaggration of *E. coli* cells.

Discussion

We found that contrasting culture conditions resulted in completely divergent impacts of *S. maltophilia* on *E. coli* biofilm formation, which was inhibited under static batch culture but greatly stimulated in continuous flow. Although biofilm formation and species interactions are expected to change with culture conditions in general (Simoes et al. 2006; Zhang et al. 2013), the turnover of interactions from antagonistic to synergistic between the same two bacteria was surprising. The result highlighted the complexity of species interactions even between two bacteria. It suggested that changing environmental conditions may convert a water bacterial strain from a helper to a repellent with regard to pathogen biofilm formation, which is critical for its survival in DWDS. Taking a step further, environmental conditions such as flow regimes may be intentionally used to control pathogens in drinking water systems.

The observed diverging interactions can be related to the specific strains selected in this study. Due to the increased curli expression (Vidal et al. 1998), the *E. coli* strain showed high autoaggregation (Figure 7) and initial attachment (Figure 5) in undisturbed, static condition. Slight disturbance due to plate shaking caused 70% reduction in its biofilm formation, suggesting that its attachment to the solid surface was fairly weak. Thus, it is not surprising that the majority (85-99%) of *E. coli* cells initially adhered during the inoculation period detached quickly under the disturbance of flow (Figure 1). In contrast, *S. maltophilia* showed more robust surface adherence than *E. coli*, as its attachment was barely affected by the same disturbances (Figure S2 and S5), and could not be challenged by the invasion of *E. coli* (Figure 6).

The opposite impacts of *S. maltophilia* on biofilm formation of *E. coli* also were attributable to different challenges for biofilm growth under the two culture conditions. Shear force was the primary challenge for biofilm growth in flow cells (Stoodley et al. 2002). Pre-attached cells need to withstand local shear force in order to remain on the surface. The surface-adherence of *S. maltophilia* was sufficiently strong to resist the shear force associated with the flow rate of 0.5 mm s^{-1} used in this study, resulting in a steady increase of biomass (Figure S2). In contrast, *E. coli* itself failed to remain on the surface in its pure culture under continuous flow. The presence of *S. maltophilia* altered the

440 circumstance, resulting a greatly expedited biofilm formation for *E. coli* in mixed-species

441 cultures. The strong surface-binding species, *S. maltophilia,* helped the poor colonizer, *E.*

442 *coli*, to attach and form biofilms, similar to the previous observations between *E. coli* and

443 other species, such as *Pseudomonas putida* (Castonguay et al. 2006) and *Pseudomonas*

444 *aeruginosa*, although the mechanisms were unclear. Co-aggregation is one of the best-

445 studied mechanisms explaining synergistic interactions among many species (Castonguay

446 et al. 2006; Klayman et al. 2009a). However, we did not observe co-aggregates of the

447 two species. Reduced local shear force by the strong colonizer was a possible explanation

448 for the protection of *E. coli* from detaching in mixed-species culture. Biofilm

449 colonization can decrease localized flow velocity near the surface to as much as 50% (de

450 Beer et al. 1994), which will reduce the shear force proportionally. Another explanation

451 is the modification of the abiotic surface by *S. maltophilia* though the production of

452 extracellular polymeric substances (Sutherland 2001) or surfactants (Castonguay et al.

453 2006), which may facilitate the adhesin recognition and attachment of *E.* coli. A third

454 explanation was unique to the cell shape of *S. maltophilia* in biofilms. Long filamentous

455 cells of *S. maltophilia* were observed in flow cells (and not in suspended culture even in

456 an extended 72-h growth) (Figure S3), which were also reported previously (Ryan et al.

457 2008). The long filaments formed a net-like matrix, which may facilitate the physical

458 trapping of *E. coli* and provide the protection to *E. coli* from being washed away.

459 In contrast, static cultures in microtiter plates differ from flow cells in many ways, which

460 may help explain the observed turnover in species interactions. Replication from

461 planktonic cells can be accumulated in microtiter plates, but hardly in flow cells.

462 Planktonic replication and cell sedimentation rather than growth from sessile cells may

463 have resulted in the formation of the thick mono-species *E. coli* biofilms in microtiter

464 plates, as reported similarly for *Legionella pneumophila* (Mampel et al. 2006). The

465 second difference relates to carbon and nutrients that are replaced continuously in flow

466 cells but depleted with time in microtiter plates. Competition with *S. maltophilia* for

467 limited substrate in batch culture resulted in less cellular growth of *E. coli* (Figure 3), and

468 thus may have contributed to the observed inhibition. However, there were still more

469 free-living *E. coli* cells in microtiter wells with mixed-species biofilms ($1.0-8.0\times10^7$ CFU

470 ml^{-1}) than that with pure *E. coli* biofilms (1.2×10^7 CFU ml^{-1}), suggesting that the

471 inhibition from *S. maltophilia* was more likely towards cell attachment rather than cell
472 growth. Thus medium replacement during culture in microtiter plates, if performed to
473 approximate nutrient supply in flow cells, may not reverse the inhibition. Moreover,
474 diffusive signals were more likely to accumulate in microtiter plates. Many signals have
475 been identified to be responsible for the competitive interactions among bacterial species
476 (Kreth et al. 2005; Ryan et al. 2008). These two factors contributed a small proportion
477 (up to 20%) to the observed inhibition on *E. coli* biofilm growth (Figure 4). The rest
478 majority of inhibition resulted from two types of *S. maltophilia* cells, free-living ones and
479 surface-attached ones. Free-living cells of *S. maltophilia* accumulated in microtiter plates
480 up to 10^9 CFU ml^{-1}. These cells can prevent planktonic *E. coli* cells from autoaggregation
481 or surface-attachment (Figures 5 and 7), and can "remove" already attached *E. coli* cells
482 (Figure 6). As a highly mobile strain (Figure S7), the swimming and twitching of the
483 10^9 free-living *S. maltophilia* per mililiter medium may introduce disturbance comparable
484 to the one caused by gentle shaking, which was shown to greatly reduce biofilm
485 formation of *E. coli* (Figure S5). Surface-attached *S. maltophilia* also prevented *E. coli*
486 from attaching in microtiter plates. Attached biomass of *E. coli* onto a surface pre-
487 covered by *S. maltophilia* was still only 23% compared to that on a naked surface after
488 excluding the impact from planktonic cells (Figure 6). Live *S. maltophilia* rather than just
489 the abiotic biofilm matrix were required for such prevention, because UV treated *S.*
490 *maltophilia* biofilm showed no inhibition to *E. coli* biofilm formation in microtiter plates
491 (data not shown).

492 Still, questions remain to fully understand the diverging interactions between *E. coli* and
493 *S. maltophilia*. Why biofilm of the same species, *S. maltophilia*, behaved so oppositely to
494 the attachment of *E. coli* in the two culturing systems may be related to different
495 morphologies, gene expressions and adhesin productions of both species. For the curli-
496 producing *E. coli* strain, its curli fimbriae are of particular importance as a mediator in its
497 interactions with *S. maltophilia*. Curli fimbriae are critical for surface anchorage and
498 multi-layer cell clustering of *E. coli* via inter-bacterial bundle formation according to a
499 previously presented biofilm model (Prigent-Combaret et al. 2000; Van Gerven et al.
500 2015). Environmental conditions including nutrient and growth phase, which differed
501 here between flow cells and batch cultures, are known to affect curli biosynthesis though

502   the curli promoter CsgD and sigma factors (Van Gerven et al. 2015). Disturbance from

503   mobile *S. maltophilia* cells as described above may impair the assembly of curli

504   monomer CsgA that takes place extracellularly, and may block the bundle formation

505   among *E. coli* cells (Prigent-Combaret et al. 2000). A difference in *S. maltophilia* biofilm

506   matrix in flow cells and in static culture may be another influential factor. Attached *S.*

507   *maltophilia* cells switched from rod cell shape to filamentous form in flow cells (Figure

508   S3), but never so in microtiter plates, even after an extended three-day culturing. Gene

509   expression and adhesin production of the same species can change significantly with

510   culture conditions, as well as when in contact with other species (Mashburn et al. 2005;

511   Jakubovics et al. 2008). Expressions of many genes can be different between the rod-

512   shaped and filamentous cells of *S. maltophilia.* One example is the filamentous

513   hemagglutinin proteins, which were shown to mediate species interactions (Ryan et al.

514   2009). Biofilm matrix composition is also expected to differ between the single layer of

515   rod-shaped *S. maltophilia* in microtiter plates and voluminous biofilms in flow cells. One

516   extracellular polysaccharide, colanic acid, is known to affect *E. coli* biofilm formation

517   (Prigent-Combaret et al. 2000). Identifying the expression and transcription of curli genes

518   of *E. coli* as well as genes and adhesins of *S. maltophilia* in co-culture is of high value

519   and may lead to the uncovering of molecular mechanisms about interactions between the

520   two species, but is beyond the scope of this study.

521   The divergent dual-species interactions in this study suggest that environmental

522   conditions need to be considered when evaluating the nature of interactions between

523   bacteria of interest. Many reactors have been used to study biofilms under simulated

524   conditions (Gomes et al. 2014). Yet, most of time only one of them was used in a

525   particular study in the literature. The nature of species interactions, e.g., synergistic or

526   antagonistic, was then concluded based on that particular culture condition. Our study

527   highlighted the value of testing different conditions such as water flow, nutrient level,

528   water chemistry, and surface material, some of which had been widely evaluated (Manuel

529   et al. 2007; Guo et al. 2013).

530   There are limitations to recognize before extrapolating our discovery in a laboratory

531   setting to realistic DWDS. For example, we selected two contrasting conditions, absolute

532   stagnancy versus uninterrupted flow to conduct this study. But flow conditions in real

533   DWDS are likely to be somewhere in between, thus the diverging effect of species

534   interactions on *E. coli* biofilm formation may be less dramatic among different sections

535   of DWDS. In addition, similar to many other studies (Simoes et al. 2007; Klayman et al.

536   2009b; Simoes et al. 2010), we used a diluted medium to conduct research about drinking

537   water related bacteria. Although diluted, these media differ from drinking water

538   especially in nutrient levels and ionic strength. Bacteria are expected to grow faster, form

539   biofilms more quickly, and reach a higher cell density in this medium than in oligotrophic

540   drinking water. It is possible that the same *E. coli* and *S. maltophilia* may behave

541   differently had they been grown in drinking water. At a minimum, it is expected that the

542   number of cells would be an order of magnitude lower in drinking water. We included a

543   10-fold lower inoculation of *E. coli* as a comparison in this study. The stimulation on its

544   biofilm formation in flow cells by *S. maltophilia* was more obvious than that with more

545   concentrated *E. coli* (Figure 1) and the inhibition in static culture was also observed

546   (Figure 2). These results suggest that the divergent effects would still be observable in

547   oligotrophic environment with less cell growth, such as in drinking water.

548   Baring these limitations, there are merits and implications in our study for pathogen

549   control in realistic scenarios. Firstly, our results strongly imply that pathogen biofilm

550   formation and its survival can differ greatly at various sections. Some are more likely to

551   become hot spots than others, depending on interactions with existing water bacteria.

552   This implication corresponds well with previous surveillance that some outbreaks

553   originated from water tanks with most likely stagnant water (Kramer et al. 1996), while

554   the others sourced from main pipes where water was flowing (Brunkard et al. 2011; Beer

555   et al. 2015). Moreover, our study highlighted additional complexity that environmental

556   conditions may pose to pathogen survival in realistic DWDS. In addition to selecting for

557   different bacteria by shaping the microbial community of water biofilms (Douterelo et al.

558   2016), environmental conditions are likely to also manipulate the relationships between a

559   pathogen and the bacterial community. Thus, the same bacterial community may be a foe

560   in one scenario, but becomes a friend in another situation. As relationships change, the

561   survival of a pathogen can be altered. This additional complexity can be used

562   intentionally for a flexible pathogen control strategy. Persisting microbial species and

563   biofilms can be managed to repel, rather than to help, the embedding of pathogens into
564   the biofilm matrix by altering environmental conditions. This probiotic approach should
565   be taken into consideration for a more effective removal of microbial contamination and
566   biofilm management plan in drinking water systems (Douterelo et al. 2016).

567   To summarize, we discovered that interactions with a water bacterium can change from
568   synergistic to antagonistic with regard to biofilm formation of a pathogen surrogate,
569   when cultured in static mode in comparison to flow mode. Similar turnover may take
570   place for the interactions between other water bacteria and contaminating pathogens in
571   real DWDS. This relationship change may be utilized purposely for effective
572   management of microbial contamination by changing environmental conditions such as
573   flow.

Acknowledgments

Conflict of interest

579   No conflict of interest declared.

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731

733   **Figure legends**

734   **Figure 1. *E. coli* biofilm growth in flow cells**

735   Growth of *E. coli* biofilms in mono-species cultures (E1, E0.1) and in mixed-species

736   cultures (E1mix, E0.1mix) in flow cells shown as (A) re-constructed 3-D images and (B)

737   quantified biomass based on confocal microscopy images. Inoculated was 1 ml 10^9 CFU

738   ml^{-1} *E. coli* (E1, E1mix) or 10-fold less, 10^8 CFU ml^{-1} *E. coli* (E0.1, E0.1mix), mixed

739   with 10^9 CFU ml^{-1} *S. maltophilia* (E1mix, E0.1mix). *E. coli* carried a constitutively

740   expressed green fluorescent protein and thus was shown as green cells in the images.

741   Images of the same row in (A) were taken at the same time-points unless specifically

742   labeled, and always from the center of flow path except where edge of flow cell was

743   indicated. Grid size is 26.7 μm. Flow cell culture systems: (●) pure culture E1; (○)

744   mixed culture E1mix; (▲) pure culture E0.1; (△) mixed culture E0.1mix

Figure 2. Biofilm growth in static microtiter plates

Mono-species (E1, E0.1, S) and mixed-species (E1mix, E1mix#, E0.1mix) biofilms were grown in static microtiter plates with plastic (A) or glass (B and C) surfaces. The biomass of biofilms was quantified with the method of crystal violet staining (A), or with the COMSTAT program based on confocal microscopy images (C). Panel B shows the reconstructed 3-D images of biofilms. Labels E1 and E0.1 indicated the inoculum of 1 μl 10^9 CFU ml^{-1} and 10-fold less, 10^8 CFU ml^{-1} *E. coli* cells, respectively, per 100 μl medium. Inoculated *S. maltophilia* was 1 μl 10^9 CFU ml^{-1} per 100 μl medium in S and E1mix, but 10-fold less, 10^8 CFU ml^{-1} in E1mix#. Error bars are standard deviations from three replicated culture (A) or from three stacks of images in the same culture (C). * and ** indicate p<0.05 in comparison to E1 and E0.1, respectively. *E. coli* carried a GFP and was shown as green cells, while *S. maltophilia* with fluorescent protein mCherry shown as red in (B). Grid size is 26.7 μm in panel B.

Figure 3. *E. coli* cell growth in planktonic pure and mixed cultures

Under the same inoculum and culture conditions as used for biofilm growth in microtiter plates (refer to Figure 2 legend), densities of *E. coli* from planktonic cultures were quantified by plate counting and shown as normalized values to that of E1. Error bars are standard deviations from three measurements. * indicates p<0.05 when comparing to E1.

Figure 4. Effect of diffusible signals on *E. coli* biofilm growth in Transwell systems

E. coli biofilms grew in a 24-well microtiter plate. Each well was coupled with a Transwell insert made of a 0.4 μm membrane, which separated cells grown in the insert from those grown in the well, but allowing diffusible signals or nutrients exchange between the well and insert. *S. maltophilia* (S) or two species mixtures (S&E) were inoculated into the inserts. Biomass of *E. coli* biofilms grown in these wells was normalized to that with no inoculation in the insert (none). Error bars are standard deviations of 3-4 replicates. * indicates p<0.05 when comparing to the 'none' control.

Figure 5. One hour initial attachment of cell suspension

E. coli (E), *S. maltophilia* (S), and their 1:1 mixed suspentions (E&S) were deposited into 96-well microtiter plates (10^9 CFU ml^{-1}, 100 μl per well) and remain static for one hour. Attached biomass was quantified and normalized to that of *E. coli* (E, as 100%). Error

775 bars are standard deviations of three replicated cultures. * indicates p<0.05 comparing to

776 E.

777 **Figure 6. Robustness of surface attachment of *E. coli* and *S. maltophilia***

778 Robustness of surface attachment was examined by invading preattached cells of one

779 species (E: *E. coli* or S: *S. maltophilia*) with suspended cells of the other species. Surface-

780 attached biomass was quantified before (□) and after (■,■) the invasion (total biomass

781 of attached invaded/invading species; planktonic cells of the invaded species were kept

782 (■) or removed (■) prior to the invasion). Biomass was normalized to that of the pre-

783 attached *E. coli* biofilm (E: set as 100%). Error bars are standard deviations among three

784 replicates. * and # indicate p<0.05 comparing the two groups.

785 **Figure 7. *E. coli* cell autoaggregation in the presence of *S. maltophilia* cells**

786 *E. coli* cells autoaggreated and settled down in static test tubes, resulting a reduction of

787 cell density in the top layer of cell suspension (24 h vs 0 h). In comparisons are *E. coli*

788 cells only (□), *E. coli* mixed with 10-fold less (△) or equal (○) numbers of *S.*

789 *maltophilia* cells. Error bars are standard deviations from three measurements.

790 ## Supporting information

791 Supplemented figures are images of mixed-species biofilms in flow cells (Figure S1),

792 biomass of *S. maltophilia* in biofilms grown in flow cells (Figure S2), filamentous cells

793 of *S. maltophilia* in flow cells (Figure S3), the effect of supernatant from *S. maltophilia*

794 cultures on *E. coli* biofilm formation (Figure S4), the effect of physical disturbance on

795 biofilm formation (Figure S5), fluorescent microscopic images of pre-grown biofilms

796 prior to and after invasion (Figure S6), and swimming and swarming motility of *E. coli*

797 and *S. maltophilia* (Figure S7). Table S1 lists component in used media in comparison to

798 in drinking water.

799 ## Supporting information legends

800 **Figure S1. Images of mixed species biofilms in flow cells.**

801 Re-constructed 3-D images of mixed-species biofilms (green-*E. coli;* red-*S. maltophilia*

802 in mixed-species cultures (E1mix, E0.1mix) showed the temporal changes in the ratio of

803 the two bacteria in biofilms within a flow cell. Grid size is 26.7 μm.

804 **Figure S2. Biomass of *S. maltophilia* in biofilms grown in flow cells.**

805 Biomass of *S. maltophilia* was quantified by COMSTAT based on confocal laser

806 scanning microscopy images (red channel only) from mixed-species cultures (E1mix: □,

807 E0.1mix: △) and the mono-species control (S: ○). The same amount of *S. maltophilia*

808 was inoculated (1 mL 10^9 CFU mL^{-1} per flow cell). Biomass was not quantified beyond

809 17 h after the start of the flow because fluorescence of mCherry severely faded.

810 **Figure S3. Filamentous cells of *S. maltophilia* in flow cells**

811 A representative white (A) and fluorescent (B) microscopy image of the *S. maltophilia*

812 mono-species biofilm cultured in the flow cell system (S) was taken at 33 h after flow

813 resumed. These images illustrate the filamentous cell morphology of *S. maltophila*. Faded

814 fluorescence of *S. maltophilia* was visible at 33 h. Rulers indicate 20 μm in length.

815 **Figure S4. The effect of supernatant from *S. maltophilia* cultures on *E. coli* biofilm**

816 **formation**

817 The supernatant harvested from *S. maltophilia* cultures at stationary or exponential

818 growth phase was supplemented with LB medium (1:1 mix by volume with 0.2× LB

819 broth) for *E. coli* biofilm cultures in static microtiter plates. The biomass of *E. coli*

820 biofilm was quantified with the method of CV staining and normalized to that in the no

821 supernatant dosing control. Error bars represent standard deviations from three replicated

822 cultures. No statistical significance was observed among tested conditions.

823 **Figure S5. The effect of physical disturbance on biofilm formation**

824 Physical disturbance was introduced to biofilm cultures in microtiter plates by gently

825 shaking the plates at 60 rpm. The relative biomass of biofilm cultured with shaking to

826 that without shaking indicated the effect of physical disturbance. Error bars represent

827 standard deviations from three replicated cultures.

828 **Figure S6. Fluorescent microscopy images of pre-grown biofilms prior to and after**
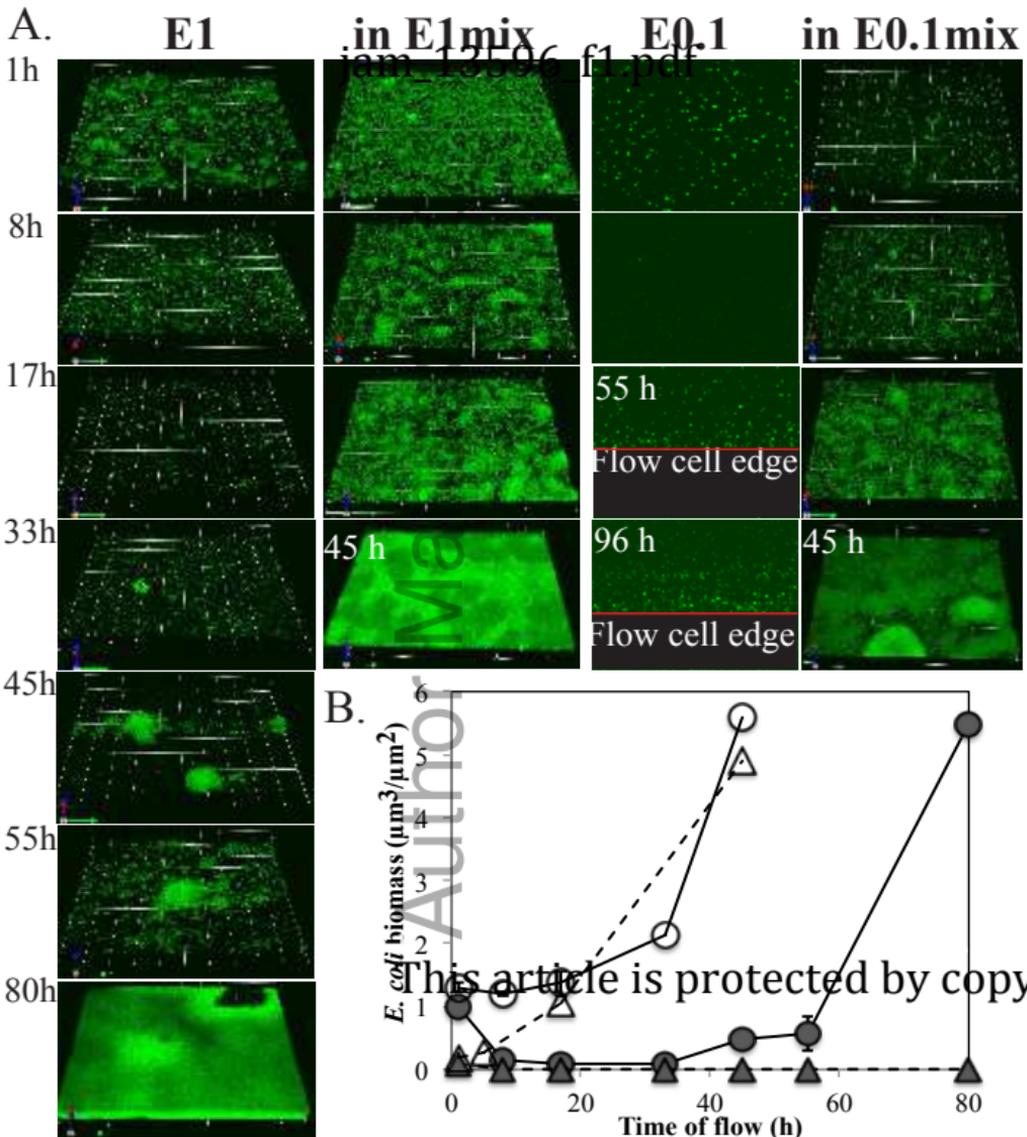
829 **invasion**

830 Biofilms of *E. coli* (A) or *S. maltophilia* (C) were pre-grown for 24 h in static microtiter

831 plates, invaded by suspended cells of the other species, and resulting biofilms were

832 imaged 24 h after the invasion (B and D). *E. coli* was shown as green or yellow cells,

833 while *S. maltophilia* was shown as red cells in the images. One representative image from

834 fluorescent microscopy was shown. Rulers indicate 20 μm in length.
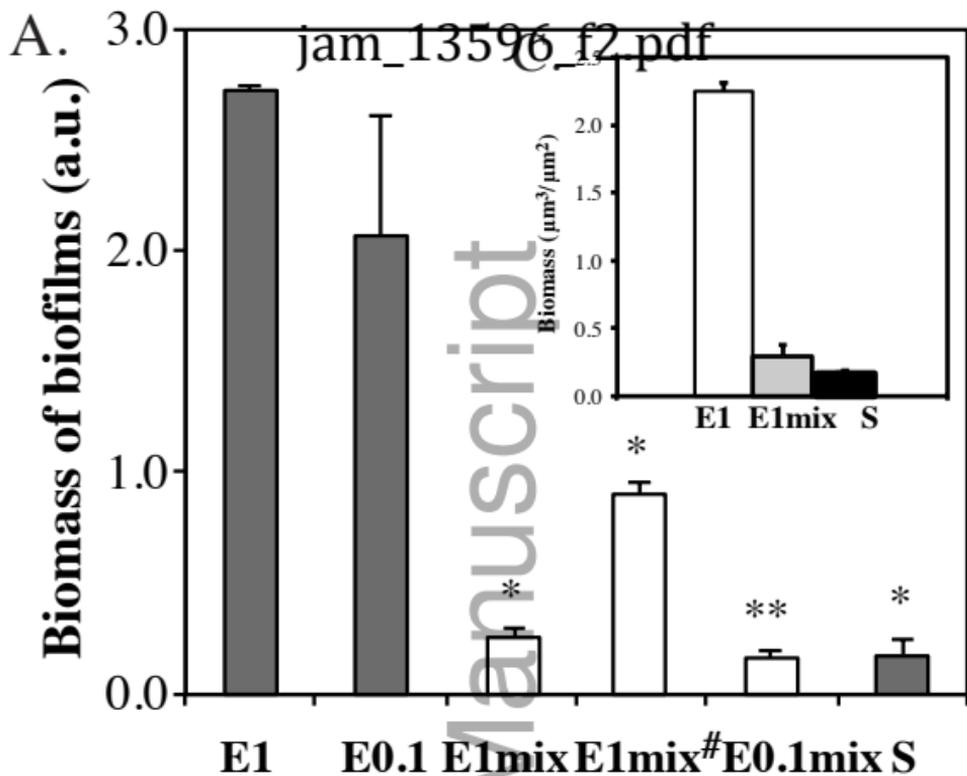
835 **Figure S7. Swimming and swarming motility of *E. coli* and *S. maltophilia***

836   The relative swimming (A) and swarming (B) motility of *E. coli* and *S. maltophilia* was

837   shown as the relative diameters of colonies on soft agar in motility test.
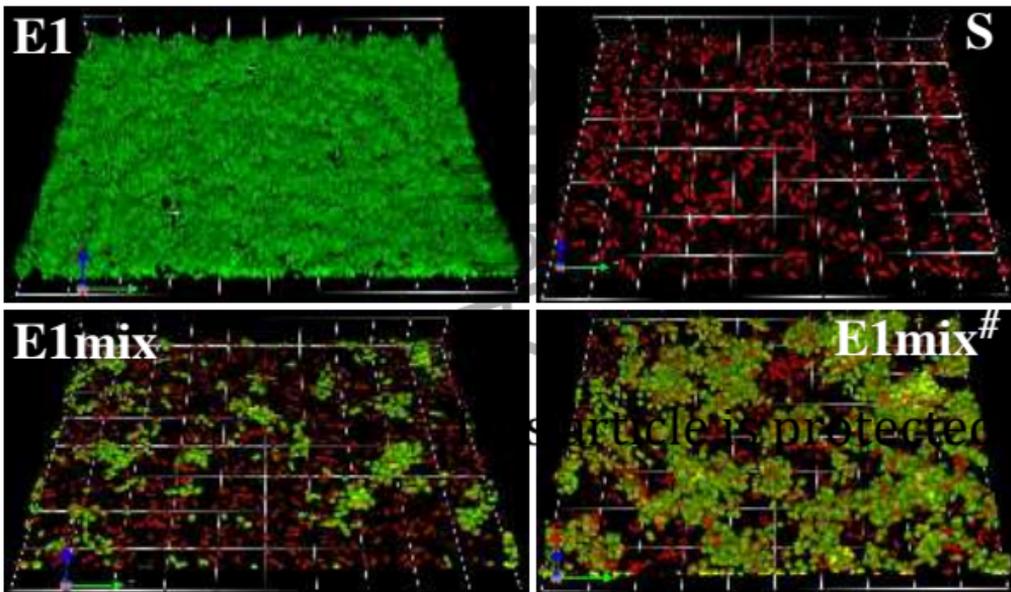
838   **Table S1. Components of culture media in comparison to drinking water**

A.

	E1	**in E1mix**	**E0.1**	**in E0.1mix**

1h

8h

17h

33h

45h

55h

80h

55 h

Flow cell edge

96 h

Flow cell edge

45 h

45 h

B.

E. coli biomass (μm³/μm²)

Time of flow (h)

A.

C.

B.

E1

S

E1mix

E1mix#

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Biomass of *E. coli* biofilm (y-axis): 0% to 100%

Cells grown in Transwell insert (x-axis): none, S, S&E
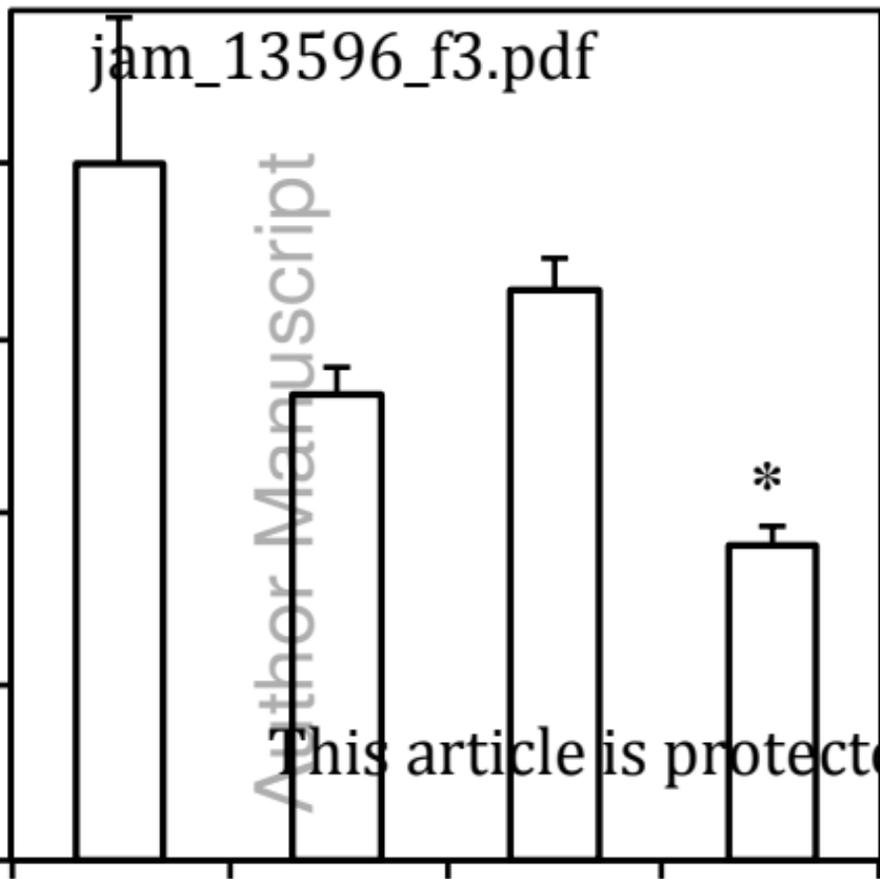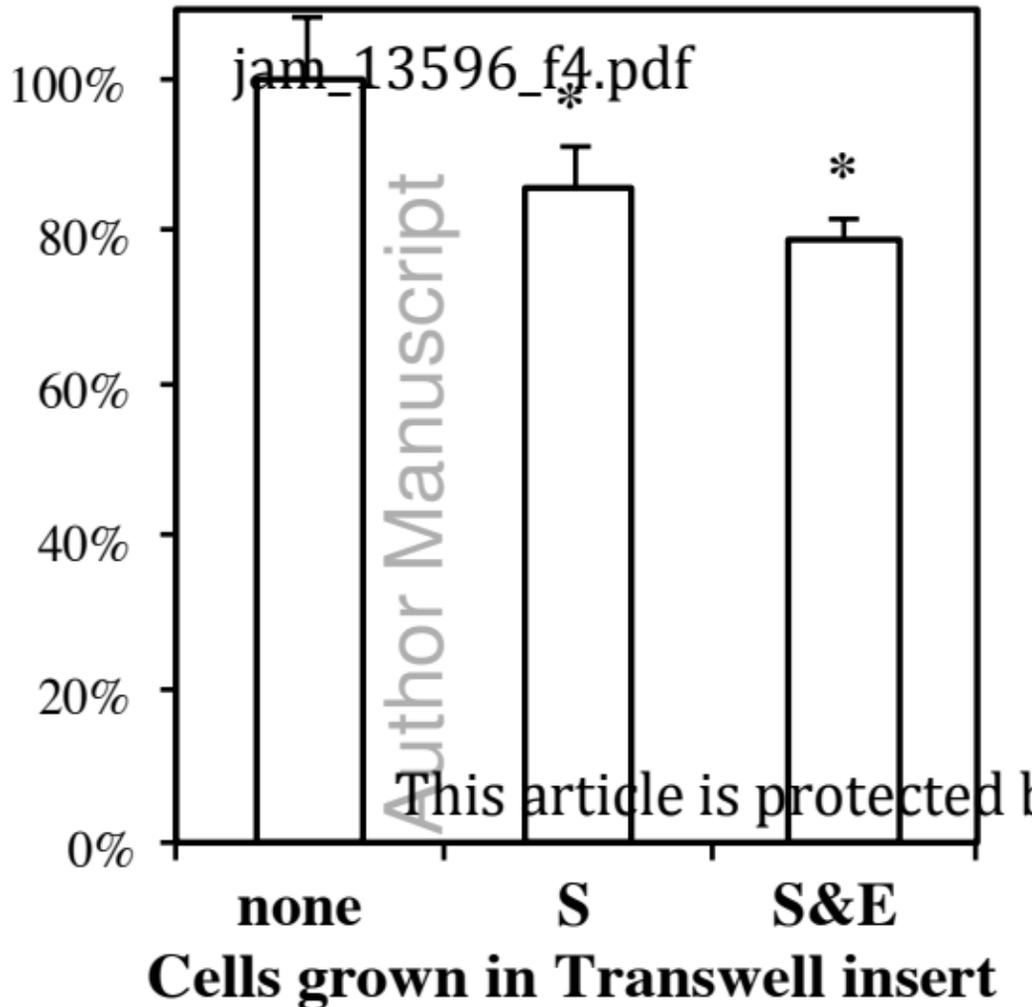
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Relative initial attachment

120%
100%
80%
60%
40%
20%
0%

E   E&S   S

*   *

This article is protecte

$E.\ coli$ cell density (CFU mL^{-1})

Time sit in test tubes

0 h   24 h

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