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ORIGINAL ARTICLE

The effect of interactions between a bacterial strain isolated from drinking water and a pathogen surrogate on biofilms formation diverged under static *vs* flow conditions

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Abstract

Aims: Interactions with water bacteria affect the incorporation of pathogens into biofilms and thus pathogen control in drinking water systems. This study was to examine the impact of static *vs* flow conditions on interactions between a pathogen and a water bacterium on pathogen biofilm formation under laboratory settings.

Methods and Results: A pathogen surrogate *Escherichia coli* and a drinking water isolate *Stenotrophomonas maltophilia* was selected for this study. Biofilm growth was examined under two distinct conditions, in flow cells with continuous medium supply *vs* in static microtitre plates with batch culture. *E. coli* biofilm was greatly stimulated (*c.* 2–1000 times faster) with the presence of *S. maltophilia* in flow cells, but surprisingly inhibited (*c.* 65–95% less biomass) in microtitre plates. These divergent effects were explained through various aspects including surface attachment, cellular growth, extracellular signals and autoaggregation.

Conclusions: Interactions with the same water bacterium resulted in different effects on *E. coli* biofilm formation when culture conditions changed from static to flow.

Significance and Impact of Study: This study highlights the complexity of species interactions on biofilm formation and suggests that environmental conditions such as the flow regime can be taken into consideration for the management of microbial contamination in drinking water systems.

Introduction

Biofilms are ubiquitous in drinking water distribution systems (DWDS) and premise plumbing (PP) (Simoes *et al.* 2006; Wang *et al.* 2014). Many waterborne disease outbreaks are linked to biofilm growth (Lau and Ashbolt 2009). Incorporation in biofilms can protect bacterial pathogens, which can be introduced through infiltration or contamination, from residual disinfectants and other harsh stresses (Wingender and Flemming 2011; Schwering *et al.* 2013; Burmolle *et al.* 2014; Sanchez-Vizuete *et al.* 2015). As a result, pathogens often survive and even proliferate in DWDS and PP (Szewzyk *et al.* 2000; Donohue *et al.* 2015). A critical factor that determines whether a pathogen can be incorporated in a biofilm is its interactions with persisting bacteria in water systems. Synergistic interactions promote its biofilm formation, while antagonistic interactions limit its embedding and growth in biofilms (Elias and Banin 2012; Burmolle *et al.* 2014; Rendueles and Ghigo 2015). Whether an interaction is synergistic, neutral or antagonistic depends on the interacting water bacteria and environmental conditions (Simoes *et al.* 2007; Klayman *et al.* 2009). Environmental factors especially hydrodynamics, and carbon/nutrient levels can modify mixed-species biofilm formation (Stoodley *et al.* 1998; Manuel *et al.* 2007; Zhang *et al.*

2013; Shen *et al.* 2015). The modification by hydrodynamics for a complex, multiple-species community can be achieved by selecting for certain bacteria against others, as microbial composition in water biofilms change with flow condition (Douterelo *et al.* 2016). Within a dual-species community, the modification may work by changing interactions between the two, as suggested by one study showing impact of flow gradients on dual-species biofilm formation (Zhang *et al.* 2013). However, it is unclear to what degree the interactions can change from flow to static conditions and whether this change will result in significant differences in pathogen survival. Although mixed-species interactions on biofilm formation have been widely investigated, surprisingly very few studies have compared this contrasting environmental condition, flow *vs* static, when evaluating dual-species interactions.

Contrasting flow conditions are relevant to pathogen control in DWDS. Various sections of DWDS, such as storage tanks and main pipes, differ greatly in water flow and corresponding nutrient replenishment. Surveillance of drinking water-related outbreaks revealed some cases of contamination originating from storage tanks (Kramer *et al.* 1996) and others from DWDS to PP pipes (Brunkard *et al.* 2011; Beer *et al.* 2015). One study particularly found that higher occurrence of coliforms was associated with DWDS containing more water tanks (LeChevallier *et al.* 1996). These reports suggest differential pathogen survival in pipes and water tanks. How contrasting flow conditions may contribute to such differences deserves systematic investigation in well controlled and replicable laboratory settings.

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

Escherichia coli is an indicator bacterium of faecal contamination in water resources. Its detection suggests the presence of pathogens originating from faecal contamination. Thus, it was used as a surrogate for pathogenic species. *Stenotrophomonas maltophilia* is frequently detected among heterotrophic plate count isolates from DWDS to PP water and biofilms (Critchley *et al.* 2003; Simões *et al.* 2007), and especially in hospital potable water (Safdar and Rolston 2007). The relative abundance of *Stenotrophomonas* spp. was reported to be 1–6% among isolates from various sampling sites in a pilot-scale DWDS (Norton and LeChevallier 2000). *S. maltophilia* is the third most common isolate (13.5%) from treated, tap, and haemodialysis water (Arvanitidou *et al.* 2003). The number of *S. maltophilia* reached 49 CFU per ml in water reservoirs of dental clinics (Szymańska 2007) and was recently detected at relatively high levels in biofilms collected from faucet aerators in 15 homes using qPCR targeting the 23S rRNA gene (Haig *et al.* 2016). It is also of clinical relevance as one of the most frequently isolated opportunistic pathogens among cystic fibrosis patients (Waters *et al.* 2011). *S. maltophilia* was thus used here to represent a persisting water bacterium. Because both *E. coli* and *S. maltophilia* have been isolated together from water systems and other environments where biofilms are important (Arvanitidou *et al.* 2003; Rudi *et al.* 2009), a scenario of their co-existence and interactions is plausible and relevant.

We aimed to evaluate the impact of contrasting flow/static conditions on dual-species interactions with regard to *E. coli* biofilm formation under laboratory settings. We studied how a drinking water bacterium *S. maltophilia* affected biofilm formation of a pathogen surrogate *E. coli* in flow cells and static plates, representing different flow conditions in drinking water systems. We further explored several aspects of biofilm formation of *E. coli,* including cell growth, surface attachment, diffusible signals and cell aggregation, in order to understand the observed difference in species interactions due to culture conditions.

Materials and methods

Bacterial strains and cultures

Escherichia coli K-12 PHL644 and *S. maltophilia* were used in this study. The *E. coli* strain is a good biofilm former due to a mutation in gene *ompR* and an increase in curli expression (Vidal *et al.* 1998). It was chosen to simulate a worse-case scenario in pathogen control where the incoming pathogens are efficient in biofilm formation by themselves. The *S. maltophilia* strain was isolated from drinking water and identified by sequencing its full-length 16S rRNA gene. Both strains were tested to be sensitive to tetracycline and gentamicin. To facilitate the differentiation of the two strains, plasmids pMP4655-GFP and

pBPF-mCherry were transformed into *E. coli* and *S. maltophilia,* respectively, by electroporation using a Gene Pulser Electroporation System (Bio-Rad, Hercules, CA, USA) following the manufacture's protocol. Transformed *E. coli* and *S. maltophilia* were selected by culturing onto Luria–Bertani (LB) agar with 40 μg ml^{-1} tetracycline or with 20 μg ml^{-1} gentamicin (Sigma-Aldrich, St. Louis, MO, USA) respectively. The constitutively expressed green fluorescence protein (GFP) in *E. coli* and red fluorescence protein (mCherry) in *S. maltophilia* were both confirmed with fluorescence microscopy. Growth rate and biofilm formation of transformed strains were not different from the ones without a plasmid (data not shown). Strains were stored in LB broth with 20% glycerol in −80°C. For active culture, strains were streaked from glycerol stocks onto LB agar with antibiotics (40 μg ml^{-1} tetracycline for *E. coli* or 20 μg ml^{-1} gentamicin for *S. maltophilia*). Single colonies from agar plates were used to inoculate broth cultures.

Broth medium was 10-fold diluted LB broth (0.1 × LB). This diluted broth was chosen to approximate oligotrophic drinking water but to still have higher levels of essential nutrients than typical drinking water to allow biofilms to grow and mature within days for laboratory study. Other media such as undiluted Reasoner's 2A (R2A) or diluted Trypticase Soy Broth (TSB) with similar nutrient levels and ionic strength as in our 0.1 × LB have been used to study biofilm formation of drinking water-related bacteria (Table S1) (Simoes *et al.* 2007, 2010; Klayman *et al.* 2009). Antibiotics were supplemented into 0.1 × LB to maintain plasmids. Broth cultures were incubated at 30°C overnight (13 h) with vigorous shaking (250 rpm). To wash off any residual antibiotics, cells of *E. coli* or *S. maltophilia* were pelleted by centrifugation (3 000×g, 3 min) and resuspended in antibiotic-free fresh 0.1 × LB for inoculation into biofilm systems. Cell densities of *E. coli* and *S. maltophilia* in inoculum were quantified by plate counting.

Biofilm cultures in flow cells

Flow systems were assembled with three-channel glass-bottom flow cells (Stovall, Greensboro, NC, USA), each channel with dimensions of 1 × 4 × 40 mm. Antibiotic-free 0.1 × LB broth was supplied at 0.12 ml min^{-1}, resulting a laminar flow (Reynolds number = 0.8) and low flow velocity (=0.5 mm s^{-1}) which is in the low range of flows in DWDS. The medium flow was paused for inoculation. One millilitre cell inoculum of *E. coli*, *S. maltophilia* or a mixture of the two was injected into each flow cell, and allowed to attach onto surfaces for 1 h. Five flow cells were run in parallel (E1, S, E1mix, E0.1, E0.1mix). Labels E1 and E0.1 represent a 10-fold difference in the inoculum of *E. coli* (10^9 CFU per ml and 10^8 CFU per ml respectively). Inoculated *S. maltophilia* was 10^9 CFU per ml in both mixed cultures (E1mix, E0.1mix) and the pure culture control (S). Medium flow was resumed and this time point was recorded as 0 h. Growth of biofilms was monitored with confocal laser scanning microscopy (details in the section of 'Imaging biofilms') at various time points until mature biofilms developed without observable change in biomass or structure or 96 h. The flow cell system was operated at 20°C and replicated independently.

Biofilm cultures in static plates

Mono- (E1, E0.1, S) and mixed-species (E1mix, E1mix$^{\#}$, E0.1mix) biofilms were grown in 96-well Nunclon microtitre plates (Fisher Scientific, Pittsburgh, PA) and in 24-well glass-bottom plates (MatTek, Ashland, MA, USA). Resuspended cells from overnight planktonic cultures were washed and inoculated into 0.1 × LB broth (100 μl per well) with no antibiotics supplemented in either pure or mixed cultures. Labels E1 and E0.1 represent an inoculum of 1 μl 10^9 CFU per ml and 10^8 CFU per ml per 100 μl medium respectively. Inoculated *S. maltophilia* was 1 μl 10^9 CFU per ml per 100 μl medium in E1mix, E0.1mix and S, but 10-fold less (1 μl 10^8 CFU per ml) in E1mix$^{\#}$. The plates were left static for biofilm growth at 20°C for 22 h. Planktonic cells in each well were gently removed and washed three times with phosphate-buffered saline (PBS, pH 7.2). Biofilm growth in a 96-well plate was quantified using a crystal violet (CV) staining method (O'Toole and Kolter 1998) and biomass was shown as OD600 (optical density at 600 nm) in arbitrary units. Four replicate cultures were grown for each type of biofilm in the same plate. Biofilm in a 24-well plate was imaged with confocal laser scanning microscopy (details in the section of 'Imaging biofilms'). Biofilm cultures were replicated three times independently.

Test the effect of diffusible signals

Planktonic cultures of *S. maltophilia* were harvested at 4 h (exponential phase) and 15 h (stationary phase) after inoculation into 0.1 × LB broth. No antibiotics were supplemented in these cultures. Supernatant was acquired by filtering planktonic cultures through membrane filters (0.22 μm, Millipore, Billerica, MA, USA). Culture medium (0.2 × LB broth) was supplemented with equal volume of the supernatant for biofilm growth in 96-well microtitre plates.

To test the impact of diffusible signals, biofilm cultures were also conducted in Transwell systems (Corning, NY). Each Transwell consists of a 24-well microtitre plate and

24 inserts, one per well. The insert has a polycarbonate membrane (0.4 μm) bottom to separate bacterial cells grown in the insert from those grown in the microtitre well, but allowing the culture medium and diffusible signals being exchanged between each pair of insert and well during the period of incubation. *E. coli* was inoculated into the microtitre wells (1 µl 10^9 CFU per ml per 100 μl medium), while *S. maltophilia* or a mixture of *E. coli* with *S. maltophilia* (equal number pool) was inoculated into the inserts (1 μl 10^9 CFU per ml per 100 μl medium). After 22 h of incubation in static at 20°C, the inserts were discarded and *E. coli* biofilms grown in the 24-well microtitre plates were quantified with the method of CV staining.

Initial attachment assay

Overnight cultures of *E. coli* and *S. maltophilia* were resuspended in fresh 0.1 × LB (no antibiotics) and adjusted to be 10^9 CFU per ml. Pure *E. coli, S. maltophilia* or their 1:1 mixtures were added into a 96-well plate (100 μl per well) and left static at 20°C for the 1-h initial attachment. Suspended cells were gently removed and washed three times with PBS. Biomass of attached cells was quantified with the method of CV staining.

Cell autoaggregation

Overnight cultures of *E. coli* and *S. maltophilia* were resuspended in PBS buffer. *E. coli* suspension, or mixed suspension of *E. coli* with 10-fold less or the same amount of *S. maltophilia* cells were prepared. Three millilitres of these suspensions were added into a 5-ml test tube (Fisher Scientific, Pittsburgh, PA, USA) and set static for 24 h. Colony-forming unit of *E. coli* in the topmost suspension column (sampled at 0 h and 24 h after setting) in the test tubes were measured by plate counting with no sample homogenization. The reduction of CFU density reflected the degree of *E. coli* cell aggregation. The experiments were repeated independently twice.

Biofilm invasion

Pure *E. coli* or *S. maltophilia* were allowed for a 1-h initial attachment in microtitre plates the same way as described above. After the 1-h initial attachment, suspended cells of this species were either gently removed or kept in the well. Suspension of the other species was added into the wells to invade the preattached species. The invasion lasted for 1 h. Attached biomass was then washed and quantified with the CV staining method. A similar invasion experiment was also performed using well-developed (24-h growth after the inoculation) rather than the 1-h pre-attached biofilms in glass-bottom plates (MatTek, Ashland, MA, USA). Biofilms before and after invasion were imaged with fluorescence microscopy (Olympus, Wirtz, VA, USA). The invasion experiments were repeated three times with similar results.

Imaging biofilms

Images of biofilms were acquired with a confocal laser scanning microscope (Fluoview™, Olympus, Wirtz, VA, USA) with filter sets for monitoring GFP and mCherry fluorescence in *E. coli* and *S. maltophilia* respectively. Images were obtained randomly from three to six spots in the centre of each flow chamber or each well of the microtitre plates. Biofilms grown near the edge of a flow chamber were acquired only if no cells were observed in the centre of a flow chamber in the pure *E. coli* culture with low inoculation (system E0.1). Three-dimensional images were reconstructed using the software Volocity 3.2 (Improvision Inc., Waltham, MA, USA) from a stack of confocal microscopy images for the x-y sections of biofilm samples. Stacks of confocal images were also analysed for biomass quantification following the manual of COMSTAT (Heydorn *et al.* 2000).

Motility test and statistics

The swimming and swarming ability of *E. coli* and *S. maltophilia* was tested on soft agar LB plates (0.3% agar for swimming and 0.5% for swarming) similar to previously described protocol (Deziel *et al.* 2001). Student t tests were performed to test whether the difference between two groups was significant ($P < 0.05$) or not.

Results

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

To investigate how *E. coli* biofilm growth was affected by *S. maltophilia*, we compared two mixed-species cultures (E1mix and E0.1mix) in flow cells with two mono-species *E. coli* cultures (E1 and E0.1) (Fig. 1). Biofilms were imaged at various time points. Three-dimensional images were constructed showing both strains (Fig. S1) or showing only *E. coli* cells (Fig. 1a) to facilitate the comparison of *E. coli* biofilm formation among different cultures. Biomass of *E. coli* or *S. maltophilia* was quantified based on microscopy images (Fig. 1b and Fig. S2).

Cells of *E. coli* initially attached onto surfaces in all flow cells during the 1 h of inoculation before flow started. However, most attached cells in mono-species systems were quickly (<5 h in E0.1) or gradually (<33 h

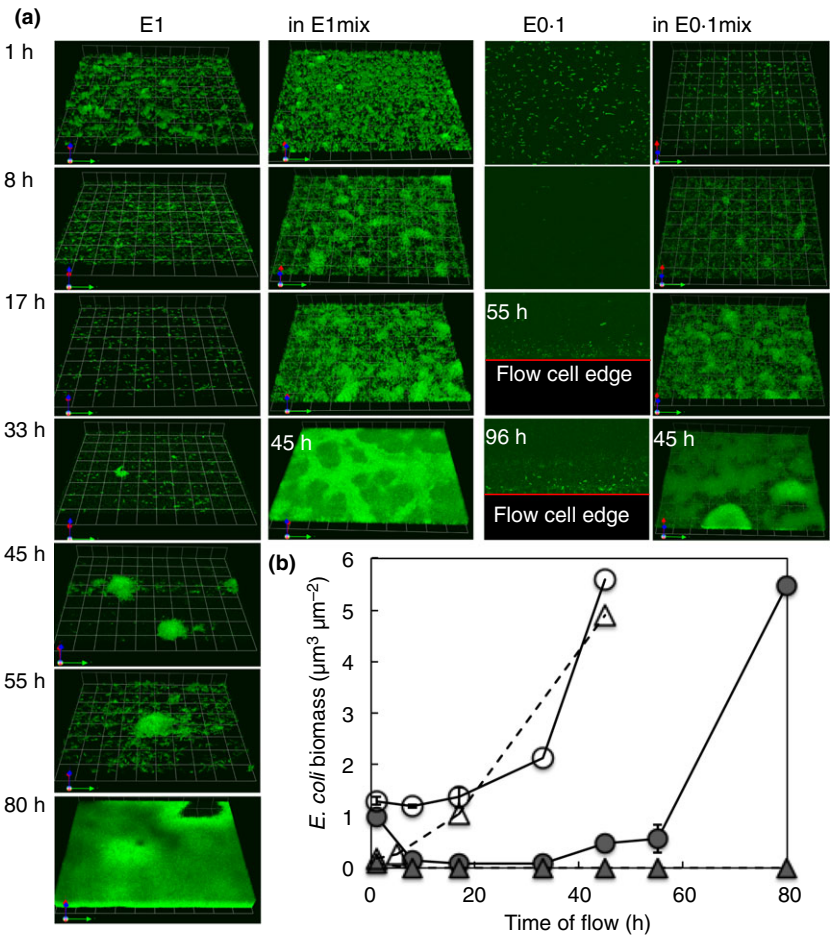

Figure 1 *Escherichia coli* biofilm growth in flow cells. Growth of *E. coli* biofilms in mono-species cultures (E1, E0.1) and in mixed-species cultures (E1mix, E0.1mix) in flow cells shown as (a) reconstructed 3D images and (b) quantified biomass based on confocal microscopy images. The following cultures were inoculated 1 ml 10^9 CFU per ml *E. coli* (E1, E1mix) or 10-fold less, 10^8 CFU per ml *E. coli* (E0.1, E0.1mix), mixed with 10^9 CFU per ml *S. maltophilia* (E1mix, E0.1mix). *E. coli* carried a constitutively expressed green fluorescent protein and thus was shown as green cells in the images. Images of the same row in (a) were taken at the same time points unless specifically labelled, and always from the centre of flow path except where edge of flow cell was indicated. Grid size is 26.7 μm. Flow cell culture systems: (●) pure culture E1; (○) mixed culture E1mix; (▲) pure culture E0.1; (△) mixed culture E0.1mix. [Colour figure can be viewed at wileyonlinelibrary.com]

in E1) washed away once flow resumed. More than 99% and 85% of *E. coli* biomass was detached from biofilms in a 7-h time period (from 1 to 8 h after starting the flow) in pure cultures E0.1 and E1 respectively. *E. coli* biomass further decreased and reached a lowest value at 33 h after starting the flow in system E1 (biomass 0.09 μm^3 μm^{-2}). Afterwards, biomass increased slightly until microcolonies grew (55 h after starting the flow) and spread until a blanket of biofilm formed 80 h after starting the flow (biomass 5.47 μm^3 μm^{-2}). In system E0.1, very few *E. coli* cells were observed in the flow cells between 55 h and 96 h after starting the flow. Some loosely attached cells were observed near the edge of the flow chamber, where shear force is close to zero (biomass <0.04 μm^3 μm^{-2}). Massive detachment of initially attached cells caused by the flow seems to be the major obstacle of *E. coli* biofilm development.

In contrast, detachment of *E. coli* was transient and much less severe in mixed-species flow cells. In E1mix, less than 10% of *E. coli* biomass was lost in a 7-h time period (from 1 to 8 h after starting the flow). *E. coli* biomass steadily increased and reached a similar level (5.59 μm^3 μm^{-2}) as in mono-species culture E1 (5.47 μm^3 μm^{-2} at 80 h) in approximately half the time (45 h, *c.* 2 times faster). The stimulation was more obvious when 10-fold less *E. coli* cells were inoculated (E0.1mix vs E0.1). The biomass of biofilms increased steadily from the first time point and reached a biomass level of 4.89 μm^3 μm^{-2} at 45 h in E0.1mix, in comparison to the no observable biofilms (<0.01 μm^3 μm^{-2}) in E0.1 by 96 h (*c.* 1000 times faster). Based on these observations, the transient and greatly reduced detachment of initially attached *E. coli* in the presence of *S. maltophilia* contributed to its expedited biofilm formation in mixed culture in flow cells.

A mono-species *S. maltophilia* flow cell (S) was run as another control. A single layer of cells was initially attached. A steady increase in biomass was observed (Fig. S2). Biomass quantification of *S. maltophilia* based on confocal microscopic images was not performed beyond 17 h after starting the flow, as accurate quantification of biomass was impossible since the fluorescent protein mCherry faded severely after 17 h. The fluorescence fading was recognized when comparing fluorescent images with white light images of biofilms within the

same scope field of view, an example of which was shown in Fig. S3. The growth of *S. maltophilia* in mixed-species biofilms was similar to its growth in mono-species culture within the first 17 h when accurate biomass quantification was available (Fig. S2). No loss of initially attached *S. maltophilia* was observed at least within the first 17 h, indicating its robust surface attachment. This solid attachment seemed to help retain *E. coli* cells on the surface, which resulted in stimulated *E. coli* biofilm growth described above.

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

Plastic microtitre plates were inoculated and incubated statically for biofilm growth with mono-species (named as E1, E0.1, and S) and mixed-species cultures (named as E1mix, E1mix$^{\#}$, E0.1mix). The biomass of biofilms (mixed- or mono-species) was quantified after 22 h of incubation. The mono-species *E. coli* biofilm had the highest biomass level of 2.73 (arbitrary unit as optical density at 600 nm) in E1, a slightly lower biomass in E0.1 (2.07), while pure *S. maltophilia* biofilm had a biomass level of only 0.17 (94% less than E1, 92% less than E0.1, $P < 0.01$) (Fig. 2a). All three mixed-species biofilms had significantly less biomass than pure *E. coli* cultures (E1 or E0.1) (biomass = 0.15–0.95, *c.* 65–95% less, $P < 0.001$), regardless of the inoculum ratios of *E. coli* and *S. maltophilia* (1:1 in E1mix, 0.1:1 in E0.1mix, and 1:0.1 in E1mix$^{\#}$). These results suggest a significant inhibition of *E. coli* biofilm formation in mixed culture, although the contribution of each species to the total biomass of mixed-species biofilms could not be determined with the CV staining method.

The observed inhibition to *E. coli* biofilm formation was independent of the surface materials when running with 0.1 × LB broth, as a similar inhibition was observed in glass microtitre plates (Fig. 2). We examined the species composition of these dual-species biofilms using microscopy images. Pure *E. coli* formed a multilayer biofilm (E1), while *S. maltophilia* (S) barely formed a single layer of cells (Fig. 2b). Mixed-species biofilms (E1mix, E1mix$^{\#}$) contained a single layer of *S. maltophilia* interspersed with microcolonies of *E. coli*, whose biomass was much less than that in E1 (quantified as *c.* 10% for E1mix, Fig. 2c). The images confirmed the inhibition on *E. coli* biofilm formation when cocultured with *S. maltophilia*.

Escherichia coli exhibited less planktonic cell growth in mixed culture than in pure culture

To identify whether the divergent effects in the flow cells *vs* the static cultures were due to cell growth differences rather than to differences related to biofilm growth, we

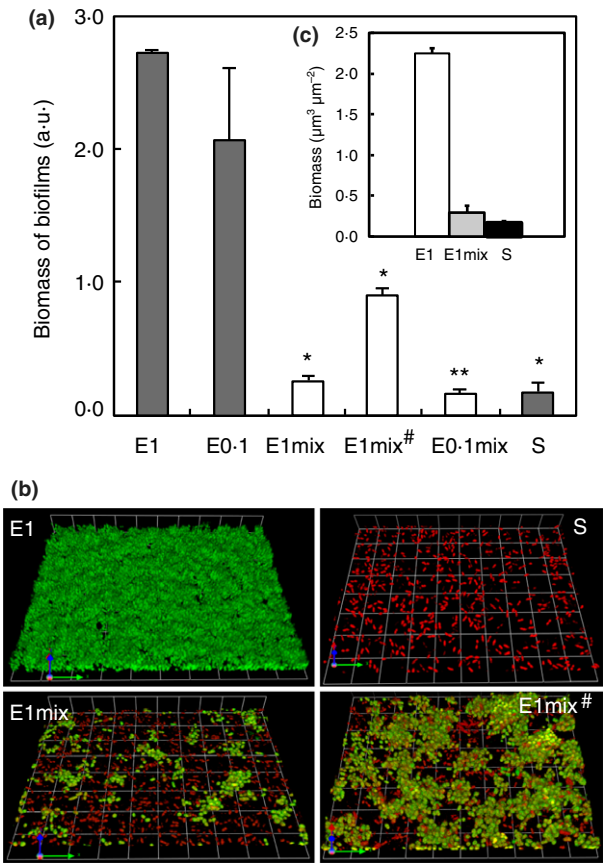

Figure 2 Biofilm growth in static microtitre plates. Mono-species (E1, E0.1, S) and mixed-species (E1mix, E1mix$^{\#}$, E0.1mix) biofilms were grown in static microtitre plates with plastic (a) or glass (b and c) surfaces. The biomass of biofilms was quantified with the method of crystal violet staining (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 per ml and 10-fold less, 10^8 CFU per ml *E. coli* cells, respectively, per 100 μl medium. Inoculated *S. maltophilia* was 1 μl 10^9 CFU per ml per 100 μl medium in S and E1mix, but 10-fold less, 10^8 CFU per ml 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). [Colour figure can be viewed at wileyonlinelibrary.com]

measured growth rates and yields of the two species in planktonic cultures. *E. coli* had a slightly lower maximum growth rate (generation time 67 ± 5 min) than *S. maltophilia* (generation time 59 ± 2 min, $P > 0.05$). We then measured yields of *E. coli* by quantifying its cell numbers in planktonic cultures with the same inoculum under the same conditions used for the biofilm cultures in microtitre plates. The number of *E. coli* cells in mixed cultures (E1mix and E1mix$^{\#}$) was 18–33% less than in

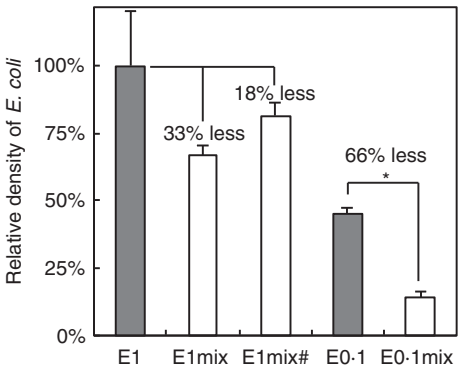

Figure 3 *Escherichia coli* cell growth in planktonic pure and mixed cultures. Under the same inoculum and culture conditions as used for biofilm growth in microtitre plates (refer to Fig. 2 legend), densities of *E. coli* from planktonic cultures were quantified by plate counting and shown as normalized values to that of E1. Error bars are standard deviations from three measurements. *indicates $P < 0.05$.

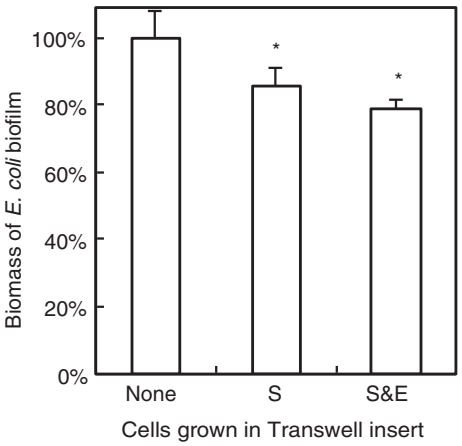

Figure 4 Effect of diffusible signals on *E. coli* biofilm growth in Transwell systems. *E. coli* biofilms grew in a 24-well microtitre plate. Each well was coupled with a Transwell insert made of a 0.4-μm membrane, which separated cells grown in the insert from those grown in the well, but allowing 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 three to four replicates. *indicates $P < 0.05$ when comparing to the 'none' control.

E. coli pure culture (E1, $P > 0.05$) (Fig. 3). The less growth of *E. coli* in mixed culture was more obvious when starting from 10-fold less *E. coli* (66% less *E. coli* in E0.1mix compared to in E0.1, $P < 0.05$).

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

To explore whether diffusible signals of *S. maltophilia* played a role in the observed divergent effects in the two systems, we harvested the supernatants of *S. maltophilia* from an exponential and a stationary growth phase and supplied them into the growth medium (1:1 mixed with 0.2 × LB broth to make it comparable with the 0.1 × LB broth) for culturing *E. coli* biofilms in microtitre plates. The biomass of *E. coli* biofilms was no different to the no supernatant control (Fig. S4). Considering that extracellular signals may have a short shelf-life after being produced, thus may be missed from the supernatant harvesting at the two predetermined time points, we used the Transwell systems (Corning, NY) to test the impact of signals produced and diffused anytime during the growth phases. These Transwell systems allowed the separation of a pure *E. coli* biofilm growth in a microtitre well from the growth of *S. maltophilia* cells or a mixed culture in the insert of that well by a 0.4-μm membrane. Signals smaller than 0.4 μm should be allowed to diffuse from the insert into the well. We observed a 14–21% decrease in *E. coli* biofilm formation in the bottom well when *S. maltophilia* or mixed-species were grown in the insert ($P < 0.05$, Fig. 4). Since nutrients and organic carbon can also freely diffuse between the inserts and wells, we cannot completely exclude the potential impact of resource competition on *E. coli* biofilm formation in these Transwell systems. Thus, diffusible signals from *S. maltophilia*, possibly combined with nutrient competition can induce up to 20% of observed inhibition on *E. coli* biofilm growth in static batch culture.

Escherichia coli exhibits weak surface attachment

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

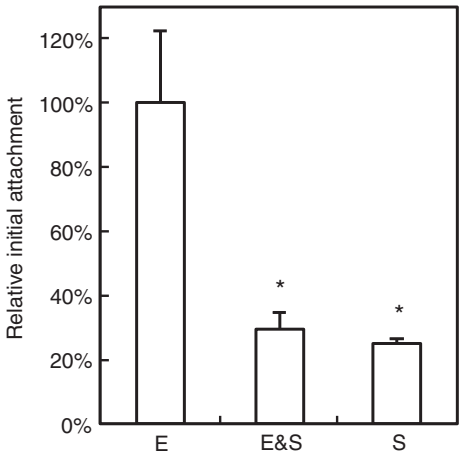

Figure 5 One-hour initial attachment of cell suspension. *E. coli* (E), *S. maltophilia* (S), and their 1:1 mixed suspensions (E&S) were deposited into 96-well microtitre plates (10^9 CFU per ml, 100 μl per well) and remain static for 1 h. Attached biomass was quantified and normalized to that of *E. coli* (E, as 100%). Error bars are standard deviations of three replicated cultures. *indicates $P < 0.05$ comparing to E.

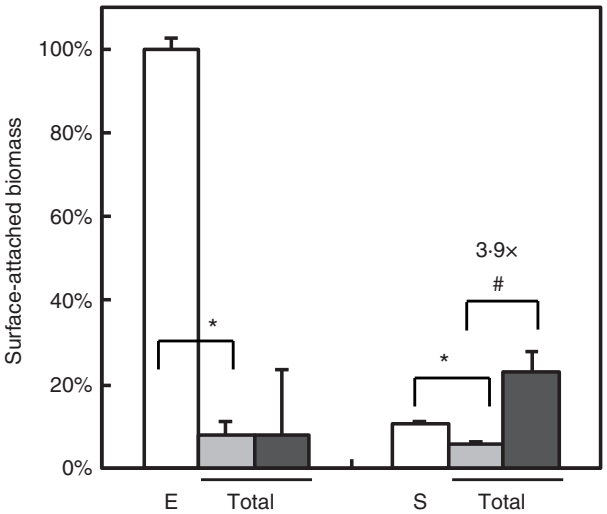

Figure 6 Robustness of surface attachment of *E. coli* and *S. maltophilia*. Robustness of surface attachment was examined by invading pre-attached cells of one species (E: *E. coli* or S: *S. maltophilia*) with suspended cells of the other species. Surface-attached biomass was quantified before (□) and after (▒,■) the invasion (total biomass of attached invaded/invading species; planktonic cells of the invaded species were kept (▒) or removed (■) prior to the invasion). Biomass was normalized to that of the pre-attached *E. coli* biofilm (E: set as 100%). Error bars are standard deviations among three replicates.
* and # indicate $P < 0.05$ comparing the two groups.

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

We further performed a series of invasion experiments to examine whether one species can outcompete the other in surface attachment. Cells of one species (invaded species) were deposited in microtitre wells for the 1-h pre-attachment. With or without removing planktonic cells of the invaded species, suspensions of the other species (invading species) were added into the wells to invade the pre-attached biofilm. About 92% of the pre-attached *E. coli* biomass was lost after the invasion by *S. maltophilia* cells (Fig. 6). It was reasonable to assume that *S. maltophilia* cells can "remove" the majority of pre-attached *E. coli* cells. The presence of planktonic *E. coli* cells showed no effect on the invasion of *S. maltophilia*. In contrast, the presence of planktonic *S. maltophilia* affected the invasion of *E. coli* to the pre-attached *S. maltophilia*. Only when planktonic *S. maltophilia* cells were removed, were the invasion of *E. coli* successful with an increased biofilm biomass, which was 3.9× more (Fig. 6). Similar results were observed when well-developed (grown for 24 h) *E. coli* biofilms on glass surface were invaded by *S. maltophilia* cells, resulting in great loss of attached *E. coli* cells and a replacement of a layer of *S. maltophilia* cells (Fig. S6). The microscopic images (Fig. S6) confirmed the assumption that attached *E. coli* can be "removed" by *S. maltophilia* cells, while in the other way, *E. coli* cells barely succeeded in attaching onto a surface which a single layer of *S. maltophilia* cells had occupied in the presence of free-living *S. maltophilia* cells.

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

Cell autoaggregation is critical for biofilm growth. The *E. coli* strain used in this study can autoaggregate due to a mutation in the *ompR* gene (Vidal *et al.* 1998). We examined how the presence of *S. maltophilia* affected the aggregation of *E. coli* cells. In a static suspension column, cells aggregated and may settle down by gravity, resulting a decreased cell density in the top layer of the column, especially when cell density was measured with the method of plate counting (one aggregate grows into one colony-forming unit, CFU). The *E. coli* cell density in the top layer showed three orders of magnitude decrease in its CFU after being static for 24 h (Fig. 7), while

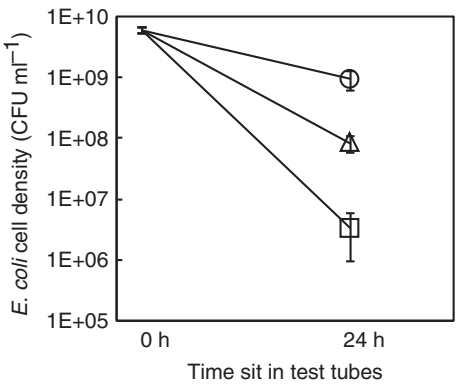

Figure 7 *Escherichia coli* cell autoaggregation in the presence of *S. maltophilia* cells. *E. coli* cells autoaggregated and settled down in static test tubes, resulting in a reduction of cell density in the top layer of cell suspension (24 h vs 0 h). In comparisons are *E. coli* cells only (□), *E. coli* mixed with 10-fold less (△) or equal (○) numbers of *S. maltophilia* cells. Error bars are standard deviations from three measurements.

S. maltophilia showed no decrease (data not shown). The decrease was alleviated to only one or two orders of magnitude when *E. coli* was mixed with an equal number or a 10-fold less numbers of *S. maltophilia* cells (Fig. 7) respectively. It indicated that planktonic *S. maltophilia* cells can reduce the autoaggregation 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 (Fig. 7) and initial attachment (Fig. 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 (Fig. 1). In contrast, *S. maltophilia* showed more robust surface adherence than *E. coli*, as its attachment was barely affected by the same disturbances (Figs. S2 and S5), and could not be challenged by the invasion of *E. coli* (Fig. 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 in biomass (Fig. 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 circumstance, resulting in a greatly expedited biofilm formation for *E. coli* in mixed-species cultures. The strong surface-binding species, *S. maltophilia,* helped the poor colonizer, *E. coli*, to attach and form biofilms, similar to the previous observations between *E. coli* and other species, such as *Pseudomonas putida* (Castonguay *et al.* 2006) and *Pseudomonas aeruginosa*, although the mechanisms were unclear. Co-aggregation is one of the best-studied mechanisms explaining synergistic interactions among many species (Castonguay *et al.* 2006; Klayman *et al.* 2009). However, we did not observe co-aggregates of the two species. Reduced local shear force by the strong colonizer was a possible explanation for the protection of *E. coli* from detaching in mixed-species culture. Biofilm colonization can decrease localized flow velocity near the surface to as much as 50% (de Beer *et al.* 1994), which will reduce the shear force proportionally. Another explanation is the modification of the abiotic surface by *S. maltophilia* via the production of extracellular polymeric substances (Sutherland 2001) or surfactants (Castonguay *et al.* 2006), which may facilitate the adhesin recognition and attachment of *E.* coli. A third explanation was unique to the cell shape of *S. maltophilia* in biofilms. Long filamentous cells of *S. maltophilia* were observed in flow cells (and not in suspended culture even in an extended 72-h growth) (Fig. S3), which were also reported previously (Ryan *et al.* 2008). The long filaments formed a net-like matrix, which may facilitate the physical trapping of *E. coli* and provide the protection to *E. coli* from being washed away.

In contrast, static cultures in microtitre plates differ from flow cells in many ways, which may help explain the observed turnover in species interactions. Replication from planktonic cells can be accumulated in microtitre plates, but hardly in flow cells. Planktonic replication and cell sedimentation rather than growth from sessile cells may have resulted in the formation of the thick mono-species *E. coli* biofilms in microtitre plates, as reported similarly for *Legionella pneumophila* (Mampel *et al.* 2006). The second difference relates to carbon and nutrients that are replaced continuously in flow cells but depleted with time in microtitre plates. Competition with *S. maltophilia* for limited substrate in batch culture resulted in less cellular growth of *E. coli* (Fig. 3), and thus may have contributed to the observed inhibition. However, there were still more free-living *E. coli* cells ($1.0–8.0 \times 10^7$ CFU per ml) in microtitre wells with mixed-species biofilms than that with pure *E. coli* biofilms (1.2×10^7 CFU per ml), suggesting that the inhibition from *S. maltophilia* was more likely towards cell attachment rather than cell growth. Thus, medium replacement during culture in microtitre plates, if performed to approximate nutrient supply in flow cells, may not reverse the inhibition. Moreover, diffusive signals were more likely to accumulate in microtitre plates. Many signals have been identified to be responsible for the competitive interactions among bacterial species (Kreth *et al.* 2005; Ryan *et al.* 2008). These two factors contributed a small proportion (up to 20%) to the observed inhibition on *E. coli* biofilm growth (Fig. 4). The rest majority of inhibition resulted from two types of *S. maltophilia* cells, free-living ones and surface-attached ones. Free-living cells of *S. maltophilia* accumulated in microtitre plates up to 10^9 CFU per ml. These cells can prevent planktonic *E. coli* cells from autoaggregation or surface attachment (Figs. 5 and 7), and can "remove" already attached *E. coli* cells (Fig. 6). As a highly mobile strain (Fig. S7), the swimming and twitching of the 10^9 free-living *S. maltophilia* per millilitre medium may introduce disturbance comparable to the one caused by gentle shaking, which was shown to greatly reduce biofilm formation of *E. coli* (Fig. S5). Surface-attached *S. maltophilia* also prevented *E. coli* from attaching in microtitre plates. Attached biomass of *E. coli* onto a surface precovered by *S. maltophilia* was still only 23% compared to that on a naked surface after excluding the impact from planktonic cells (Fig. 6). Live *S. maltophilia* rather than just the abiotic biofilm matrix were required for such prevention, because UV-treated *S. maltophilia* biofilm showed no inhibition to *E. coli* biofilm formation in microtitre plates (data not shown).

Still, questions remain to fully understand the diverging interactions between *E. coli* and *S. maltophilia*. Why biofilm of the same species, *S. maltophilia*, behaved so oppositely to the attachment of *E. coli* in the two culturing systems may be related to different morphologies, gene expressions and adhesin productions of both species. For the curli-producing *E. coli* strain, its curli fimbriae are of particular importance as a mediator in its interactions with *S. maltophilia*. Curli fimbriae are critical for surface anchorage and multilayer cell clustering of *E. coli* via interbacterial bundle formation according to a previously presented biofilm model (Prigent-Combaret *et al.* 2000; Van Gerven *et al.* 2015). Environmental conditions including nutrient and growth phase, which differed here between flow cells and batch cultures, are known to affect curli biosynthesis through the curli promoter CsgD and sigma factors (Van Gerven *et al.* 2015). Disturbance from mobile *S. maltophilia* cells as described above may impair the assembly of curli monomer CsgA that takes place extracellularly, and may block the bundle formation among *E. coli* cells (Prigent-Combaret *et al.* 2000). The difference in *S. maltophilia* biofilm matrix in flow cells and in static culture may be another influential factor. Attached *S. maltophilia* cells switched from rod cell shape to filamentous form in flow cells (Fig. S3), but never so in microtitre plates, even after an extended 3-day culturing. Gene expression and adhesin production of the same species can change significantly with culture conditions, as well as when in contact with other species (Mashburn *et al.* 2005; Jakubovics *et al.* 2008). Expressions of many genes can be different between the rod shaped and filamentous cells of *S. maltophilia.* One example is the filamentous haemagglutinin proteins, which were shown to mediate species interactions (Ryan *et al.* 2009). Biofilm matrix composition is also expected to differ between the single layer of rod-shaped *S. maltophilia* in microtitre plates and voluminous biofilms in flow cells. One extracellular polysaccharide, colanic acid, is known to affect *E. coli* biofilm formation (Prigent-Combaret *et al.* 2000). Identifying the expression and transcription of curli genes of *E. coli* as well as genes and adhesins of *S. maltophilia* in coculture is of high value and may lead to the uncovering of molecular mechanisms about interactions between the two species, but is beyond the scope of this study.

The divergent dual-species interactions in this study suggest that environmental conditions need to be considered when evaluating the nature of interactions between bacteria of interest. Many reactors have been used to study biofilms under simulated conditions (Gomes *et al.* 2014). Yet, most of the time only one of them was used in a particular study in the literature. The nature of species interactions, for example, synergistic or antagonistic, was then concluded based on that particular culture condition. Our study highlighted the value of testing

different conditions such as water flow, nutrient level, water chemistry and surface material, some of which had been widely evaluated (Manuel *et al.* 2007; Guo *et al.* 2013).

There are limitations to recognize before extrapolating our discovery in a laboratory setting to realistic DWDS. For example, we selected two contrasting conditions, absolute stagnancy *vs* uninterrupted flow to conduct this study. But flow conditions in real DWDS are likely to be somewhere in between, thus the diverging effect of species interactions on *E. coli* biofilm formation may be less dramatic among different sections of DWDS. In addition, similar to many other studies (Simoes *et al.* 2007, 2010; Klayman *et al.* 2009), we used a diluted medium to conduct research about drinking water-related bacteria. Although diluted, these media differ from drinking water especially in nutrient levels and ionic strength. Bacteria are expected to grow faster, form biofilms more quickly and reach a higher cell density in this medium than in oligotrophic drinking water. It is possible that the same *E. coli* and *S. maltophilia* may behave differently had they been grown in drinking water. At a minimum, it is expected that the number of cells would be an order of magnitude lower in drinking water. We included a 10-fold lower inoculation of *E. coli* as a comparison in this study. The stimulation on its biofilm formation in flow cells by *S. maltophilia* was more obvious than that with more concentrated *E. coli* (Fig. 1) and the inhibition in static culture was also observed (Fig. 2). These results suggest that the divergent effects would still be observable in oligotrophic environment with less cell growth, such as in drinking water.

Baring these limitations, there are merits and implications in our study for pathogen control in realistic scenarios. Firstly, our results strongly imply that pathogen biofilm formation and its survival can differ greatly at various sections. Some are more likely to become hot spots than others, depending on interactions with existing water bacteria. This implication corresponds well with previous surveillance that some outbreaks originated from water tanks with most likely stagnant water (Kramer *et al.* 1996), while the others sourced from main pipes where water was flowing (Brunkard *et al.* 2011; Beer *et al.* 2015). Moreover, our study highlighted additional complexity that environmental conditions may pose to pathogen survival in realistic DWDS. In addition to selecting for different bacteria by shaping the microbial community of water biofilms (Douterelo *et al.* 2016), environmental conditions are likely to also manipulate the relationships between a pathogen and the bacterial community. Thus, the same bacterial community may be a foe in one scenario, but becomes a friend in another situation. As relationships change, the survival of a pathogen can be altered. This additional complexity can be used intentionally for a flexible pathogen control strategy. Persisting microbial species and biofilms can be managed to repel, rather than to help, the embedding of pathogens into the biofilm matrix by altering environmental conditions. This probiotic approach should be taken into consideration for a more effective removal of microbial contamination and biofilm management plan in drinking water systems (Douterelo *et al.* 2016).

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

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Images of mixed-species biofilms in flow cells. Reconstructed 3-D images of mixed-species biofilms (green—*E. coli;* red—*S. maltophilia* in mixed-species cultures (E1mix) showed the temporal changes in the ratio of the two bacteria in biofilms within a flow cell. Grid size is 26.7 μm

Figure S2 Biomass of *S. maltophilia* in biofilms grown in flow cells. Biomass of *S. maltophilia* was quantified by COMSTAT based on confocal laser scanning microscopy images (red channel only) from mixed-species cultures (E1mix: □, E0.1mix: △) and the mono-species control (S: ○). The same amount of *S. maltophilia* was inoculated (1 ml 10^9 CFU per ml per flow cell). Biomass was not quantified beyond 17 h after the start of the flow because fluorescence of mCherry severely faded

Figure S3 Filamentous cells of *S. maltophilia* in flow cells. A representative white (a) and fluorescent (b) microscopy image of the *S. maltophilia* mono-species biofilm cultured in the flow cell system (S) was taken at 33 h after flow resumed. These images illustrate the filamentous cell morphology of *S. maltophila*. Faded fluorescence of *S. maltophilia* was visible at 33 h. Rulers indicate 20 μm in length

Figure S4 The effect of supernatant from *S. maltophilia* cultures on *E. coli* biofilm formation. The supernatant harvested from *S. maltophilia* cultures at stationary or exponential growth phase was supplemented with LB medium (1:1 mix by volume with 0.2 × LB broth) for *E. coli* biofilm cultures in static microtitre plates. The biomass of *E. coli* biofilm was quantified with the method

of CV staining and normalized to that in the no supernatant dosing control. Error bars represent standard deviations from three replicated cultures. No statistical significance was observed among tested conditions

Figure S5 The effect of physical disturbance on biofilm formation. Physical disturbance was introduced to biofilm cultures in microtitre plates by gently shaking the plates at 60 rpm. The relative biomass of biofilm cultured with shaking to that without shaking indicated the effect of physical disturbance. Error bars represent standard deviations from three replicated cultures

Figure S6 Fluorescent microscopy images of pregrown biofilms prior to and after invasion. Biofilms of *E. coli* (a) or *S. maltophilia* (c) were pregrown for 24 h in static microtitre plates, invaded by suspended cells of the other species, and resulting biofilms were imaged 24 h after the invasion (b and d). *E. coli* was shown as green or yellow cells, while *S. maltophilia* was shown as red cells in the images. One representative image from fluorescent microscopy was shown. Rulers indicate 20 μm in length

Figure S7 Swimming and swarming motility of *E. coli* and *S. maltophilia.* The relative swimming and swarming motility of *E. coli* and *S. maltophilia* was shown as the relative diameters of colonies on soft agar in motility test

Table S1 Components of culture media in comparison to drinking water.