# Invasibility of resident biofilms by allochthonous communities in bioreactors

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## 1. Introduction

Communities of macro- or microorganisms in many environments, e.g. prairies or wastewater treatment plants, are constantly exposed to the presence of non-native organisms. Those non-native organisms that are able to establish themselves, spread and consequentially invade the local communities are defined as invasive species (Litchman, 2010). Invasion by non-native or allochthonous species may pose serious threats to biodiversity, human health, food security and the economy (Pimentel et al., 2000). On the other hand, the directed invasion of species with a specific function or trait (i.e. bioaugmentation) has the potential to significantly improve a desired ecosystem function. The great interest of predicting and controlling invasion for the management of ecosystem services has led to intensive research activities on what determines the failure or success of an invasive species (Fargione and Tilman, 2005; Strayer, 2012). However, most of the studies focused on macro-organism ecosystems while less work has been done on microbial ecosystems.

Biofilms deserve attention as the majority of the microbial life is organized in these spatially structured habitats (Battin et al., 2007; Hall-Stoodley et al., 2004). Biofilms are attached to a surface and embedded in a self-produced matrix, composed of extracellular polymeric substance. Biofilms are used to the benefit of humans in a plethora of engineered environments, including water and wastewater treatment, remediation of contaminated soil and groundwater, but they also play a role in pathogenic infections of macro organisms, and biofouling of industrial facilities.

Several studies attempted to understand the mechanisms driving the invasion of biofilms by allochthonous organisms (Burmølle et al., 2006; Iasur-Kruh et al., 2011; Jackson et al., 2001; Zhao et al., 2006), but the conclusions are inconsistent. The ability of an invader to adhere and clolonise the native communities has a pivotal role (Russo et al., 2015), but the true drivers of the success of invasion seem to be related to the characteristics of the resident

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community, such as diversity and evenness, competition for resources, as well as changes in environmental conditions (van Elsas et al., 2012; Iasur-Kruh et al., 2011; Burmølle et al., 2006; Guillier et al., 2008; Mejlholm and Dalgaard, 2007; Zhao et al., 2006). A negative correlation between soil microbial diversity and the survival of *Escherichia coli* O157:H7 was reported by van Elsas et al. (2012). Similarly, a mixed biofilm of four bacteria isolated from marine algae was more resistant to the invasion by the antibacterial protein-producing *Pseudoalteromonas tunicata* in comparison to mono-species biofilms. The cooperative interaction between the residents of the mixed biofilm provided a protection against the invasion by *P. tunicata*, as well as the diffusion of antimicrobial agents (Burmølle et al., 2006). In wetland ponds, the amount of nutrients supplied determined the effectiveness of invasion by promoting coexistence of species, including an introduced allochthonous estradiol-degrading bacterium which increased the removal of estradiol from the systems (Iasur-Kruh et al., 2011). Yet, perturbations (i.e. antibiotic treatment) of the native community seem to facilitate the colonization and spreading of pathogens like *Candida albicans* (Huppert et al., 1953). Besides the mentioned biotic and abiotic factors regulating invasion of biofilms, other mecha-nisms such as predation and the genetic pool of the resident com-munity could be responsible for the fate of invader species (Bouchez et al., 2000; Habimana et al., 2009). Indeed, all these studies help to develop microbial management strategies to favor or limit invasi-bility. However, one *caveat* of the studies so far is the focus on the interaction between one invasive species and native communities of varying diversity. In water and wastewater treatment, monospecies invasion events are unlikely as both types of systems are challenged with potentially invasive complex microbial consortia contained for example in raw sewage or detached biofilm particles from water piping. The use of a complex community as source of allochthonous organisms may reflect more realistically the situation in engineered systems. This approach may also favour invasion because in-teractions between members of the invading community (e.g. co-aggregation) are possible.

In contrast to previous studies, here we investigated how the exposure of native communities to a complex allochthonous community changed the community structure of the resulting biofilm. We use qPCR and Capillary Electrophoreses Single-Strand Conformation Polymorphisms (CE-SSCP) as microbial community fingerprinting tool for quantifying changes in abundance and composition of developing biofilm communities after exposure to potential invaders. For CE-SSCP analysis, the 16S rRNA gene of the total bacterial pool is first amplified by Polymerase Chain Reaction (PCR). The double-stranded DNA is then denatured. In a capillary, the migration behaviour of refolded single stranded DNA molecules is characteristic of its DNA sequences. CE-SSCP has been demonstrated to detect changes in community structure at a reasonable resolution even in complex environments such as anaerobic digesters (Zumstein et al., 2000).

Biofilms were grown in bubble column reactors and analysed before and after the exposure to (1) an invading community in the form of a second mature biofilm on transferred surfaces or (2) a suspension of activated sludge. The aim of our work was to assess the ability of members of the allochthonous consortia to notably shift the development of the resident communities following the invasion events, as well as its competition for space.

## 2. Materials and methods

### 2.1. Experimental design and reactor set up

Three custom-made bubble column reactors with an inner diameter of 86 mm and a height 130 cm were set up in parallel for the growth of biofilms (Fig. 1). Each reactor had a working volume of 5 L and was supplied with a constant air flow of 3 L/min from the bottom through a neoprene membrane. The selected airflow rate guaranteed quasi-instantaneous mixing, oxygen concentrations at near-saturation and constant wall shear stress. The reactors were partially submerged into a water bath to maintain a constant temperature of 28.5 ± 0.5 °C. The pH in the systems was routinely measured and equaled 8.5.

Two reactors, W1 and W2, were inoculated with tap water (250 ml) and substrate (4.75 L). The third reactor, S, was inoculated with 250 ml of activated sludge from the aeration tank of a municipal wastewater treatment plant (Vinassan, France) diluted in 4.75 L of substrate to achieve a final concentration of total suspended solid (TSS) and volatile suspended solid (VSS) equal to ca. 235 mg/L and 192 mg/l, respectively. The substrate was composed

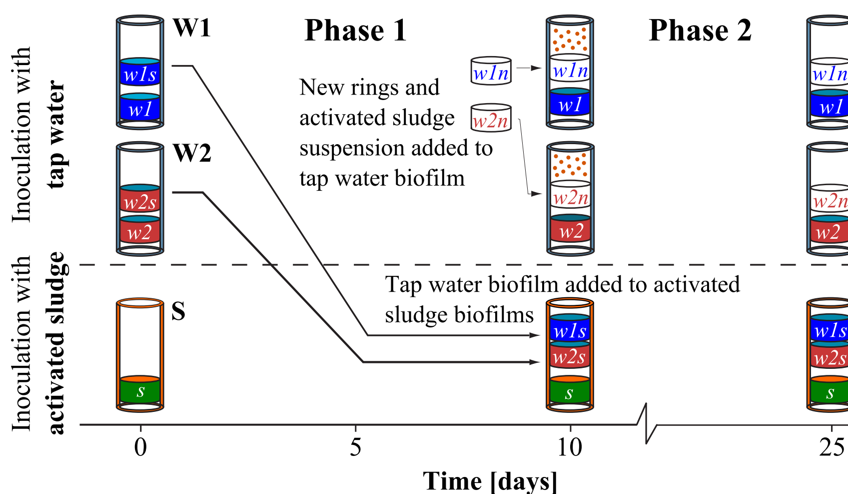


**Fig. 1.** Schematic diagram of the reactor set up and experimental design. W1 and W2 were inoculated with tap water while S was inoculated with fresh activated sludge. At time 0 rings named, *w1* and *w1s*, were inserted in W1, *w2* and *w2s* in W2, and *s* in S. The system runs for 10 days in order to deveolop mature biofilm (Phase 1). At day 10, rings *w1s* and *w2s* were transferred into reactor S, whereas rings *w1n* and *w2n* that contain clean coupons were inserted into W1 and W2, respectively. At the same time, suspension of fresh activated sludge, which represents the invading mixed consortia, was pumped in W1 and W2. After that, the reactors run for additional 15 days (Phase 2).

of 0.21 g/L of meat extract, 0.21 g/L of yeast extract, 0.21 g/L of peptone, 0.009 g/L of $NH_4Cl$, 0.03 g/L of $K_2HPO_4$, 0.015 g/L of $KH_2PO_4$, 0.003 g/L of $MgSO_4$ $7H_2O$, 0.003 g/L of $CaCl_2$ $2H_2O$ and 0.03 g/L of $Na_2HPO_4$ $2H_2O$. The substrate was sterilized by autoclaving. Five milliliters of sterile-filtered trace element solution (1.75 ml/l of $FeCl_3$, 0.075 g/L of $H_3BO_3$, 0.015 g/L of $CuSO_4$ $5H_2O$, 0.09 g/L of KI, 0.038 g/L of $MnCl_2$, 0.03 g/L of $Na_2MoO_4$ $2H_2O$, 0.06 g/L of $ZnSO_4$ $7H_2O$, 0.075 of $CoCl_2$ $6H_2O$, 0.5 ml/L of EDTA, 12 ml/L of (1N) Hydrochloric acid) and 0.03 g/L of $NaHCO_3$ were added.

Stainless steel rings (OD 83 mm, ID 79 mm, height 50 mm) were inserted in the water column of each reactor as shown in Fig. 1. The biofilm grew on polyethylene coupons (188 μm × 50 mm × 10 mm; ibidi GmbH Integrated BioDiagnostics, Martinsried, Germany) glued on the inside of the stainless steel rings with Master MS PRO (Master-In, Cagnes sur Mer, France). A sufficient number of coupons to last for the entire experiment were initially attached to the rings. This strategy allowed us to remove coupons during sampling without having to replace them. At the start of the reactor operation, the rings labeled *w1* and *w1s*, and *w2*, *w2s*, were inserted into reactors W1 and W2, respectively. Ring *s* was inserted in reactor S. The reactors were operated in batch for 24 h before switching to continuous mode. The hydraulic retention time during continuous operation mode was 65 min. This relatively short retention time and the removal of bulk phase at the top and the bottom of the column enabled us to minimize the development and accumulation of suspended biomass in the bulk phase from detached and incoming microorganisms. Each of the three reactors were continuously fed with a mixture of partially softened tap water at a flow-rate of 75 ml/min and a five times more concentrated substrate solution as before at 0.5 ml/min including 1 ml/L of trace element solution and 0.03 g/L of $NaHCO_3$. The trace element solution and the $NaHCO_3$ were added aseptically to the autoclaved substrate. The composition of the nutrient solution was modified slightly from Zhang and Bishop (2001), that was known to favour growth of complex biofilms. On day 10, rings *w1s* and *w2s* with matured biofilm were transferred from reactors W1 and W2 to S (Fig. 1), representing an invading biofilm community. At the same time, suspensions of 250 ml of fresh activated sludge diluted in 250 ml of tap water (final VSS equal to ca. 1900 mg/L) were pumped into reactors W1 and W2. The suspension was added at a flow rate of 4 ml/min in addition to the normal flow of tap water and substrate solution at 75.5 ml/min. By continuously pumping activated sludge into the reactors over a period of two hours, we ensured that a significant and constant amount of activated sludge biomass was present in reactors W1 and W2 for a period that typically allows irreversible attachment of suspended cells. The overall mass of added activated sludge roughly equaled the amount of biomass used to inoculate reactor S at day 0 (final VSS of the inoculum ca 192 mg/L). By considering the dry weight of one cell equal to $2.8 \times 10^{-13}$ g (Pepper et al., 2014) we can estimate that the total number of invading microbes added into each reactor was around $3.4 \times 10^{12}$ cells, neglecting any contribution of EPS or multicellular organisms. At the same time, the number of cells in the invading community roughly equaled the estimated total biomass of $2.6 \times 10^{11}$ cells present in the reactor system at the time of the invasion event. The estimate was done using qPCR results for biomass estimation in the biofilm.

At day 10, new rings, called *w1n* and *w2n*, with virgin coupons were also inserted into W1 and W2, respectively. Control reactors in a strict sense were not operated. If there had been an immediate and marked effect of disturbing the tap water biofilm by confronting it with an activated sludge suspension, a control reactor in a stricter sense would have been required.

After the invasion events, the reactors were operated for another 15 days (Phase 2). The total time of the reactor run was 25 days. During the experiment, every two days a ring was temporarily removed from each reactor and one coupon per ring was collected, cut in half (to have duplicate materials) and transferred into 500 μl of sterile molecular grade water before being stored at −20 °C. Once the coupons were detached from the rings, the rings were put back into the reactors. Sampling took less than two minutes during which the biofilms remained fully hydrated. During sampling, aeration and inflow of feeding solution (tap water and nutrients) were switched off.

### 2.2. DNA extraction

DNA was extracted from the biofilm grown on the surface of one-half of a coupon (2.5 $cm^2$). To promote cell lysis and biofilm detachment, a preliminary heat treatment and bead beating step was performed according to Rochex et al. (2008) before using the QIAamp DNA mini kit (QIAGEN, Courtaboeuf, France) for DNA extraction. The purity and amount of the extracted DNA was checked using an Infinite NanoQuant M200 (Tecan Group Ltd., Männedorf, Switzerland). The extracted DNA was stored at −20 °C until further analyses.

### 2.3. Quantitative PCR (qPCR)

The number of copies of the 16S rRNA gene of the bacterial biofilm community was quantified by qPCR. The universal primers BAC338F (5′ − ACTCC TACGG GAGGC AG − 3′) and BAC805R (5′ − GACTA CCAGG GTATC TAATC C − 3′) (Yu et al., 2005) targeting the 16S rRNA gene were used in a 25 μl PCR mixture containing 4.5 μl of MilliQ, 12.5 μl of Express qPCR Supermix with premixed ROX (Invitrogen, France), 10 nM of each primer, 8 nM TaqMan probe BAC16F (5′ − TGCCA GCAGC CGCGG TAATA C-3′) (Yu et al., 2005), and 5 μl of extracted DNA.

The qPCR program included an initial incubation of 20 s at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 1 min were performed. Each run was performed in duplicates using a Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany). The number of bacteria was calculated by considering an average of 4.2 copies of the 16S rRNA gene per cell (Klappenbach et al., 2001).

### 2.4. PCR and CE-SSCP

Molecular fingerprints of the samples were compared by capillary electrophoreses Single-Strand Conformation Polymorphisms (CE-SSCP). This analysis required the amplification of the V3 region of the bacterial 16S rRNA gene by PCR using the following primer pair: 5′-ACGGTCCAGACTCCTACGGG-3 (forward primer, *E. coli* position 331) and 5′-TTACCGCGGCTGCTGGCAC-3 (reverse primer, *E. coli* position 533) labelled at the 5′ end with fluorescein phosphoramidite. The PCR reactions mixture contained 37.5 μl of sterile MilliQ water, 5 μl of 10 × pfu turbo buffer, 4 μl of 2.5 mM dNTP, 1 μl each primer (final concentration 8 nM), 0.5 μl of 2.5 U/μl Stratagene PfuTurbo DNA Polymerase (Stratagene, La Jolla, CA, USA) and 1 μl of extracted DNA. PCRs were performed using a Mastercycler (Eppendorf AG, Hamburg, Germany) with the following program: denaturation for 2 min at 94 °C, 25 cycles at 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 10 min (Rochex et al., 2008). The correct size of the PCR amplicons was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Fingerprint analyses of the biofilm communities were performed by analyzing the amplified PCR products in a 3130 genetic analyzer (AB applied biosystems, Carlsbad, CA, USA) using Capillary Electrophoresis-Single Strand Conformation Polymorphisms (CE-SSCP) according to a protocol previously described by Rochex et al.

(2008). A mixture of ROX internal size standards were added to each sample. CE-SSCP profiles were then aligned using the ROX standards, baseline-adjusted and normalized using R and the package StatFingerprints (Michelland and Cauquil, 2010; Michelland et al., 2009; R Development Core Team, 2013). Successively, a semi-automated binning procedure was used to assign peaks into bins. This step allowed a more robust comparison between profiles by eliminating slight alignment errors. The average bin size was adapted to minimize false positive detection of differences between profiles, i.e. assigning peaks belonging to the same organism into two bins. At the same time, the risk of leveling differences between profiles (i.e. by putting peaks belonging to different organisms into the same bins) was low as the CE-SSCP profiles were not saturated.

### 2.5. *Statistical analysis*

All statistical analyses were performed using R version 3.0.2 (R Development Core Team, 2013) and the R package vegan 2.0–10 (Oksanen et al., 2013). In multiple-pairwise comparisons between CE-SSCP profiles, we analysed (a) the composition (i.e. the presence/absence of peaks in the fingerprints) and (b) the community structure (i.e. presence/absence of peaks and peak heights) of the biofilm samples. The comparisons were done using the Raup and Crick (RC) and Jaccard (J) metrics, calculated by functions raupcrick {vegan} and vegdist{vegan}. The two metrics differ mainly in that the Raup and Crick metric only considers presence/absence while the quantitative version of the Jaccard metric as calculated in vegdist{vegan} additionally considers abundance information. The range of both metrics is between 0 and 1. The higher the value of any of the metrices, the more different the two profiles are.

The values of the Raup and Crick metric can be considered as the probability that the compared samples have a non-identical species composition. This probability is calculated based on a pairwise comparison of the two considered profiles with 999 simulated profiles that were randomly constructed: do two observed profiles share significantly more peaks than randomly constructed profiles? Peaks for the randomly constructed profiles are drawn from the metacommunity of peaks from all CE-SSCP profiles that were analysed. The probability of drawing a peak is scaled by the relative abundance of the peak in the metacommunity. We consider non-differences in composition between two profiles as significant when the RC value falls below 0.05. At values greater than 0.95, differences in composition between profiles are considered significant.

Two-dimensional Principal Coordinate Analysis (PCoA) was done using cmdscale{stats} in R using both Jaccard and Raup and Crick distance matrices. Trajectories of community development were compared using protest{vegan}, a permutation test for Procrustes superposition analysis. We used the Jaccard and Raup and Crick distance matrices to evaluate the convergence/divergence of the communities with different biofilm histories over time.

## 3. Results

Three bubble column reactors had been operated in parallel for 25 days (Fig. 1) with a mixture of unsterile tap water and nutrient solution as a continuous influent. Two of the reactors (W1 and W2) were inoculated with tap water and one, reactor S, with fresh sludge from a municipal wastewater treatment plant. The community structure of the activated sludge was by far more complex in terms of numbers of peaks in the CE-SSCP community fingerprints than the tap water inoculum (see day 1 in Figs. S1, S2 and S3 in the Supplementary data).

Biofilm developing on the reactor surfaces could be analysed on removable, single-use, polyethylene coupons that were glued on stainless steel rings and put in place at the beginning of the reactor operation. The two rings in reactor W1 were labelled *w1* and *w1s*. Rings in W2 were labelled *w2*, *w2s*, while the ring in reactor S was labelled *s*. After 10 days of operation, *w1s* and *w2s* were moved to reactor S to investigate the exchange between mature biofilms originating from different sources, namely activated sludge and tap water. Suspensions of fresh activated sludge were pumped into W1 and W2 after adding new rings, *w1n* and *w2n*, into W1 and W2, respectively. This was done to assess the ability of the allochthonous consortia to colonize and spread into the mature biofilms formed in *w1* and *w2*, and/or its competition for space with the resident community in *w1n* and *w2n*.

### 3.1. *Biofilm growth*

Biofilm development was measured over time by qPCR and plotted in Fig. 2. Total bacterial abundances ranged between $5.7 \times 10^6$ and $4.1 \times 10^9$ cells/cm$^2$. No significant differences were found in the number of bacteria, irrespective of biofilm age and initial type of inoculum (all p-values > 0.05, analysis of variance (ANOVA) with biofilm age and inoculum type as factors). Similar counts of cells were also found in biofilms taken before and after the addition of the activated sludge suspensions in reactors W1 and W2 and the biofilm communities on *w1s* and *w2s*, which were developed in W1 and W2 and then transferred into S (all p-value > 0.05, ANOVA).

### 3.2. *Bacterial community dynamics*

Principal Coordinate Analysis (PCoA) using the Jaccard and Raup

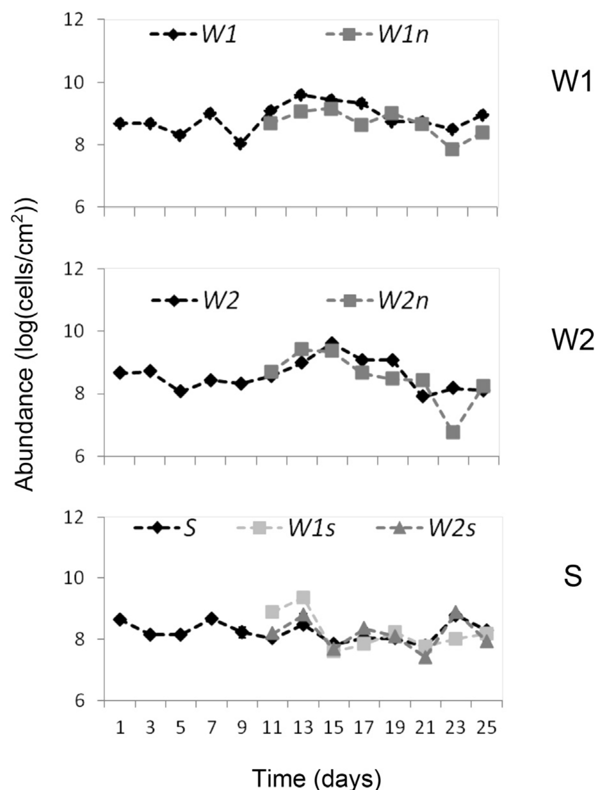


**Fig. 2.** Abundance of the total bacteria in the biofilms grown in reactors W1, W2, and S over time assessed by qPCR. The different points give average values of two replicates for the various rings in the reactors.

and Crick metrics showed that early biofilm samples in reactors W1 and W2 were similar in community structure (community composition and abundance distribution) as they occupy the same region of the ordination plot (Fig. 3A, B, D, E). In reactor S, the profiles of the microbial communities grouped together at day 1, 3 and 9, but they converged with those found in W1 and W2 at days 5 and 7 (Fig. 3C and F). During these days, the community in reactor S faced a decrease in complexity suggesting that the majority of the activated sludge community was not able to gain dominance against the microbes coming from the continuous flow of unsterile tap water used to dilute the influent. On day 9, we observed, however, an increase in complexity in the maturing biofilm in reactor S (see Fig. S3).

After the addition of activated sludge suspension to reactors W1 and W2 and mature biofilms (on rings *w1s* and *w2s*) to S, communities on all rings within one reactor followed similar developmental trajectories as revealed by protest analysis (see Fig. 3).

### 3.3. Comparing biofilm structure of same age

Pairwise comparisons were done between CE-SSCP profiles of biofilms collected on the same sampling day. This allows assessing (1) the reproducibility of duplicate reactors and rings over time (*w1* and *w2*, *w1n* and *w2n*, *w1s and w2s*) (Fig. 4A), (2) the discrepancies between the mature and early stage biofilms in W1 and W2 after the addition of the activated sludge suspension at day 10 (*w1* and *w1n*, and *w2* and *w2n*) (Fig. 4B), (3) the convergence/divergence between the invading biofilm communities on *w1s* and *w2s* and the autochthonous community on *s* in reactor S (Fig. 4C), and (4) the differences on the biofilm developed in reactors inoculated from differing sources (Fig. 4D).

To distinguish whether these changes in the communities were caused by the appearance/disappearance of peaks in the CE-SSCP profiles or by changes in the abundance distribution of peaks, pairwise differences between communities were calculated using Jaccard and Raup and Crick metrics. The Jaccard metric compares at the same time community composition and the abundance distribution while the Raup and Crick metric only considers presence/absence information. Using the two metrics, we are able to discern whether changes in community composition or in the abundance distribution contribute strongest to observed changes over time. If the Raup and Crick analysis cannot detect differences in the community, while the Jaccard metric indicates changes, we conclude that the difference in the community structure is related to the abundance information and not the composition. This interpretation is only possible when considering the two types of analysis as done here.

The inocula (tap water and fresh activated sludge) used in the reactors were composed of differing microbial communities and promoted the development of biofilms with different complexity as shown in Fig. 4D (Phase 1, day 9). In agreement with the PCoA results, also the comparison of the CE-SSCP profiles of *w1* and *w2* with *s* revealed a convergence in the community composition during days 5 and 7. During Phase 2 both the composition (RC up to

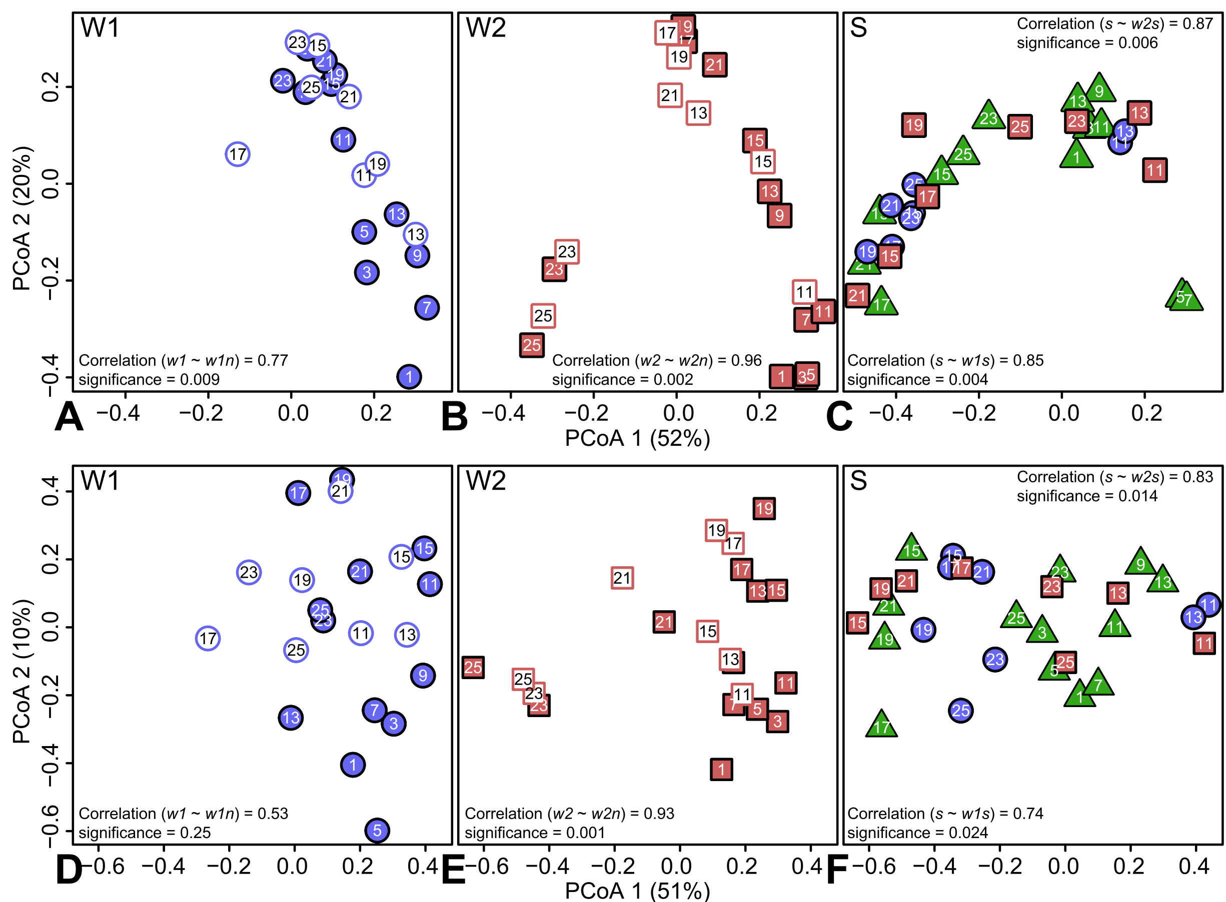


**Fig. 3.** PCoA analyses based on Jaccard dissimilarity (A–C) and Raup and Crick (D–F) index over time. The PCoA analysis was done using the entire data set, but the results were sorted by reactors for easier visualisation. Numbers in symbols indicate sampling days. Development of biofilms in (A, D) reactor W1 on rings *w1* (filled circles) and *w1n* (empty circles), (B, E) reactor W2 on rings *w2* (filled squares) and *w2n* (empty squares) and (C, F) reactor S on rings *s* (triangles), *w1s* (circles) and *w2s* (squares). Correlations and significance values result from Protest comparison of developmental trajectories between the indicated rings by time point.

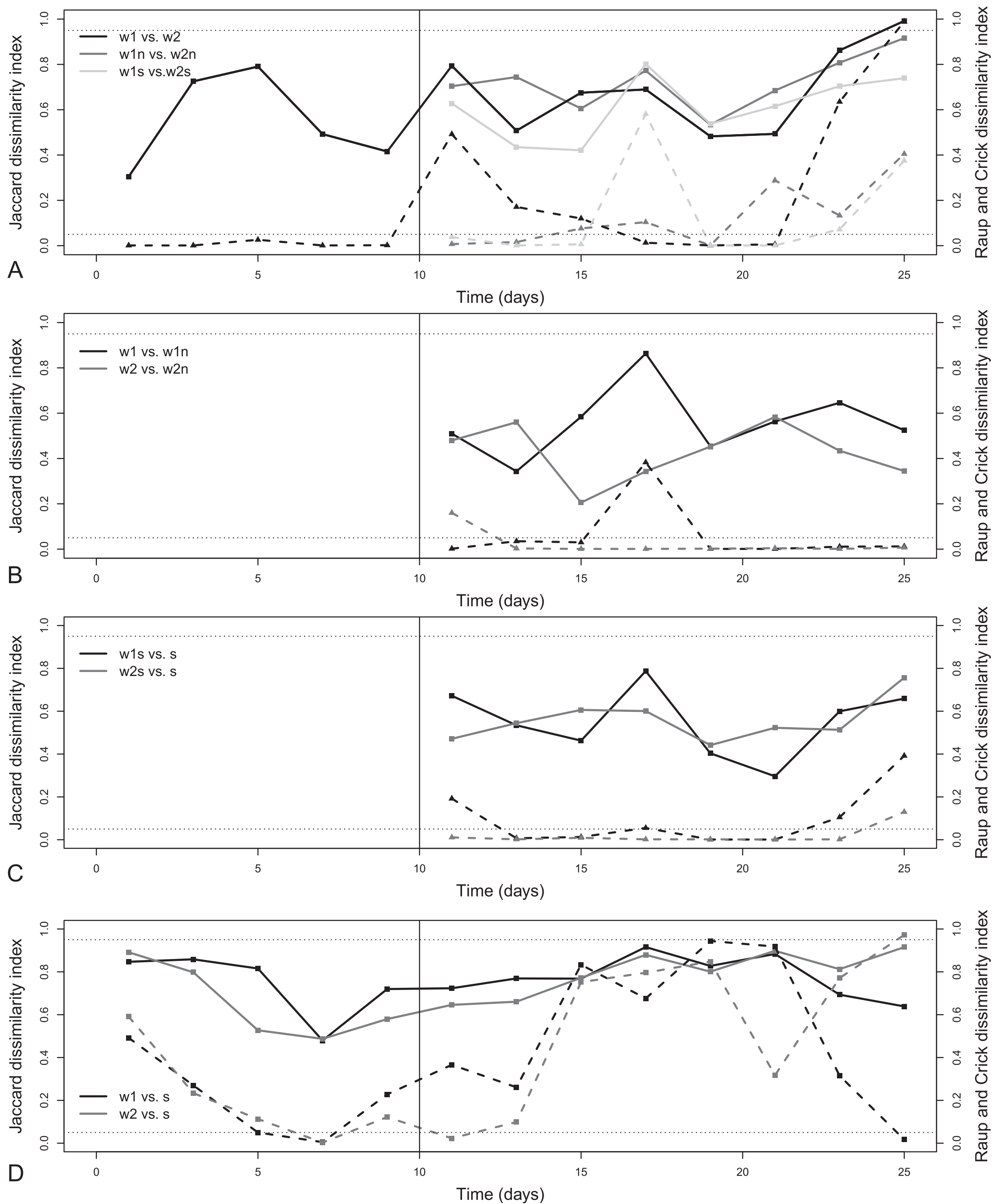


**Fig. 4.** Jaccard (solid lines; abundance and composition) and Raup and Crick (dash lines; composition) dissimilarity indeces of CE-SSCP biofilm profiles taken the same day from putative replicate systems in reactor W1, W2, and S (A), between early stage and mature biofilms (B), among biofilm communities developed in reactor S (C), and between biofilms developed in reactors inoculated with tap water, W1 and W2, and wastewater, S, (D). Raup and Crick values below 0.05 and above 0.95 (dotted lines) are indicative of significant similarity and dissimilarity, respectively, between the two biofilm communities.

0.95) and the abundance distribution of the main peaks in the profiles (J up to 0.9) indicate increasing dissimilarity between *w1*/*w2* and *s*. This suggests that the inocula and the operation (invading procedure) of the two types of systems had effects on the biofilm dynamics. However, it is interesting to note a complete convergence between *w1* and *s* in composition (low RC) but not in the abundance distribution, and an opposite trend between *w2* and *s* (high RC and high J) at day 25. The similarity between the communities in W1 and S might indicate a colonisation of the established community in *w1* by members of the activated sludge suspensions added in W1.

Notable divergence in the abundance distribution of the community members was observed also by comparing the profiles of biofilms belonging to replicated reactors and rings (*w1 vs. w2*, *w1n vs. w2n*, *w1s vs. w2s*) ($J > 0.4$). The composition of the biofilms was similar at the beginning of the run but diverged at the end of the study as the RC values in each set of comparison increased over time (Fig. 4A). The discrepancy became more evident at the end of the study (after day 23) suggesting that long term behaviour of biofilms is difficult to reproduce even in duplicate reactors.

Convergence of the communities in mature biofilm originating from tap water (*w1s* and *w2s*) and activated sludge (*s*) occurred also

in reactor S during the first 10 days after the transfer of rings *w1s* and *w2s*. As shown in Fig. 4C, only the abundance of the main peaks of the profiles (divergent J), but not the composition (similar RC), changed indicating that the resident biofilms immediately colonised the transferred coupons. Nevertheless at the end of the reactor operation, also the composition on the biofilms originating from the activated sludge and the tap water became less similar as shown by the increased values of RC at days 23 and 25. Probably few members of the original tap water biofilms in *w1s* and *w2s* managed to survive and grow up in the system.

Similar biofilm composition (RC lower than 0.05), but with fluctuating species abundance ($J > 0.4$) were obtained when comparing biofilms developed on coupons which were inserted in the reactors at the beginning of the experiment and the biofilms on new surfaces after 10 days (*w1 vs. w1n* and *w2 vs. w2n* in Fig. 4B). However, values for J within one reactor were generally lower than those between replicates (p-values $< 0.05$; ANOVA), indicating that the biofilm communities within the system rather than reactor configuration shaped the biofilm formation and evolution on the new coupons.

## 4. Discussion

Our study represents one of the few studies investigating the invasion of multispecies biofilms by mixed microbial communities. The goal was to gain insights into the mechanisms occurring between the established communities and the allochthonous species continuously entering for example wastewater treatment plants and/or drinking water pipes. To achieve this, resident biofilm was exposed to allochthonous complex microbial communities and the degree of invasion into the resulting community was monitored. We used a suspension of fresh activated sludge to invade a native biofilm derived from a tap water inoculum (reactors W1 and W2). At the same time, we investigated whether tap water biofilm communities invade a native biofilm originating from an activated sludge inoculum (reactor S).

During Phase 1, biofilms in reactors W1 and W2 were composed uniquely of individuals from the tap water community. In reactor S, a complex biofilm composed of microbes originating from both activated sludge and tap water developed as suggested by the increase convergence of the community fingerprints of from *w1*/*w2* and *s* in Fig. 4D and Fig. 3. The presence of tap water organisms in reactor S was caused by the exposure to a suspended tap water community at low levels, passing through the system as a continuous flow of unsterilized dilution water. Matured biofilms from the two inocula were then exposed to allochthonous communities in Phase 2, i.e. suspended activated sludge or mature tap water biofilm on transferred surfaces.

The results suggest that immediately after the invasion event, an established biofilm in a bioreactor outcompetes allochthonous communities for newly available surfaces, whether these are colonized or not. Transferred biofilms or newly developed biofilms rapidly adopted the community composition of the resident biofilms (low RC), with a different abundance distribution (moderate values for J) as shown in Fig. 4B and C. It is likely that the microbial community within the system owned a strong competitive advantage as already acclimatized to the operating conditions. Likewise, it may be that the resident biofilm and its corresponding detached members in the bulk phase outnumbered the invading community and therefore dominated the colonization of newly available surface. It should be noted, however, that the amount of invaders was roughly estimated to be equal the total biofilm biomass in the system. Nevertheless, in agreement with a similar study on stream biofilm communities (Besemer et al., 2012), the microbial community on the new coupons was not randomly assembled by a community made up of invaders (activated sludge) and resident (tap water) communities, but it was shaped mainly from the original community in the system.

However, the dominance of the resident biofilm on the allochthonous communities seems to become ancillary with time. After three weeks into the experiment (days 23 and 25) evidence of putative invasion of native biofilms by the allochthonous communities arose, but in different ways. The high convergence between the composition of the biofilms developed in reactors S and W1 towards the end of the experiment suggest that the activated sludge community that was added to W1 eventually had its effect on the communities on *w1* and *w1n* (low RC values in Fig. 4D) giving rise to a mixed tap water and activated sludge community, similar to the biofilms in S. A sudden decrease in biodiversity occurred in W2, where one peak largely dominated the community fingerprints (see Fig. S2). A peak at the same position in the CE-SSCP electropherogram of the dominant peak in *w2* gained relative importance also in *s*, though the community remained more diverse. The appearance of this signature peak in reactors W2 and S, and the lack of this peak in W1 let us conclude that a contamination of the dilution water reservoir can be disregarded. This reservoir was the only shared input to the reactors, as all three systems were otherwise entirely independently operated. It is plausible that the appearance of this peak documents a successful invasion of a member from the activated sludge community that grew up and became apparent only after a latency of almost two weeks. For most of this period, the number of individuals of this microbe was under the detection limit in the highly dense biofilm community. This idea is further supported by the first appearance of the peak in the S communities at day 15, comparable to the latency after the exposure to activated sludge in W2. However, we cannot rule out that this community member arrived with the transferred ring *w2s* and thus originates from the tap water biofilm in W2.

Also in reactor S, the dominant effect of the native community in *s* on the tap water biofilms decreased with time. As shown in Fig. 4B and A, the biofilms in *w1s* and *w2s* started to diverge from the resident community at day 25. The comparisons of the CE-SSCP profiles of the biofilms in the three rings (Fig. S3) demonstrated that while the community in *s* remained quite complex, few peaks dominated the community in *w1s* and *w2s*. Contrarily to what occurred in W1 and W2, it seems that some of the original members of the tap water biofilms survived the initial colonization of the resident community of S. They remained latent for 15 days before blooming. Which members regain influence does not seem to follow an apparent pattern.

It is interesting to note that over the length of the study, biofilms within a bioreactor were more similar to each other than communities of attempted replicates (Fig. 4A and B). This was irrespective of whether resident biofilms, biofilms on newly colonized surfaces or biofilms on transferred surfaces were considered. Communities between replicates showed similarities comparable to those observed within a reactor only over the first nine days of the experiment (Fig. 4A, Phase 1). After this time, the biofilm communities diverged both in composition and in relative abundance of the main species within the community, with a most noticeable difference after about three weeks (Fig. 4B). Apparently, the physical separation of the two reactors sufficed to enrich specific biofilm communities, even when the physico-chemical environment in both reactors was macroscopically identical. These findings confirm the difficulty of reproducing long-term behaviour in biofilm reactors that was already pointed out by Lewandowski et al. (2004).

In conclusion, a resident multi-species biofilm community may be beneficial for the system as it equalizes the short-term effect of invading microorganisms. Nevertheless, a complete prevention of

invasion may not be achieved. We highlighted this with the emergence of putative activated sludge peaks in the community profiles of tap water biofilm approximately two weeks after exposure to the allochthonous activated sludge community.

## 5. Conclusions

- Resident communities within a reactor may have favourable effects when invasion of a system by undesired microbes needs to be prevented.
- However, the establishment of a small number of undesired microbes, undetected in the high density of resident cells in a biofilm, may be sufficient to cause a bloom of these organisms after a latency period has passed.
- Maintaining reproducible complex communities in replicate reactors remains a challenge.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found online.

## References

Battin, T.J., Sloan, W.T., Kjelleberg, S., Daims, H., Head, I.M., Curtis, T.P., et al., 2007. Microbial landscapes: new paths to biofilm research. Nat. Rev. Micro 5, 76–81.

Besemer, K., Peter, H., Logue, J.B., Langenheder, S., Lindstrom, E.S., Tranvik, L.J., et al., 2012. Unraveling assembly of stream biofilm communities. ISME J. 6, 1459–1468.

Bouchez, Patureau, Dabert, Juretschko, Doré, Delgenès, et al., 2000. Ecological study of a bioaugmentation failure. Environ. Microbiol. 2, 179–190.

Burmølle, M., Webb, J.S., Rao, D., Hansen, L.H., Sørensen, S.J., Kjelleberg, S., 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl. Environ. Microbiol. 72, 3916–3923.

Fargione, J.E., Tilman, D., 2005. Diversity decreases invasion via both sampling and complementarity effects. Ecol. Lett. 8, 604–611.

Guillier, L., Stahl, V., Hezard, B., Notz, E., Briandet, R., 2008. Modelling the competitive growth between Listeria monocytogenes and biofilm microflora of smear cheese wooden shelves. Int. J. Food Microbiol. 128, 51–57.

Habimana, O., Meyrand, M., Meylheuc, T., Kulakauskas, S., Briandet, R., 2009. Genetic features of resident biofilms determine attachment of *Listeria monocytogenes*. Appl. Environ. Microbiol. 75, 7814–7821.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Micro 2, 95–108.

Huppert, M., Macpherson, D.A., Cazin, J., 1953. Pathogenesis of *Candida albicans* infection following antibiotic therapy. I. The effect of antibiotics on the growth of *Candida albicans*. J. Bacteriol. 65, 171–176.

Iasur-Kruh, L., Hadar, Y., Minz, D., 2011. Isolation and bioaugmentation of an estradiol-degrading bacterium and its integration into a mature biofilm. Appl. Environ. Microbiol. 77, 3734–3740.

Jackson, C.R., Churchill, P.F., Roden, E.E., 2001. Successional changes in bacterial assemblage structure during epilithic biofilm development. Ecology 82, 555–566.

Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: the Ribosomal RNA Operon Copy number Database. Nucleic Acids Res. 29, 181–184.

Lewandowski, Z., Beyenal, H., Stookey, D., 2004. Reproducibility of biofilm processes and the meaning of steady state in biofilm reactors. Water Sci. Technol. 49, 359–364.

Litchman, E., 2010. Invisible invaders: non-pathogenic invasive microbes in aquatic and terrestrial ecosystems. Ecol. Lett. 13, 1560–1572.

Mejlholm, O., Dalgaard, P., 2007. Modeling and predicting the growth of lactic acid bacteria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*. J. Food Prot. 70, 2485–2497.

Michelland, R., Cauquil, L., 2010. StatFingerprints: StatFingerprints. R Package Version 2.0.

Michelland, R.J., Dejean, S., Combes, S., Fortun-Lamothe, L., Cauquil, L., 2009. StatFingerprints: a friendly graphical interface program for processing and analysis of microbial fingerprint profiles. Mol. Ecol. Resour. 9, 1359–1363.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB et al. (2013). http://cran.r-project.org/web/packages/vegan/vegan.pdf.

Pimentel, D., Lach, L., Zuniga, R., Morrison, D., 2000. Environmental and economic costs of nonindigenous species in the United States. BioScience 50, 53–65.

Pepper, I.L., Gerba, C.P., Gentry, T.J., 2014. Environmental Microbiology. Accademic Press, Elsevier, San Diego, USA.

R Development Core Team, 2013. Austria: the R Foundation for Statistical Computing: Vienna.

Rochex, A., Godon, J.-J., Bernet, N., Escudié, R., 2008. Role of shear stress on composition, diversity and dynamics of biofilm bacterial communities. Water Res. 42, 4915–4922.

Russo, P., Iturria, I., Mohedano, M.L., Caggianiello, G., Rainieri, S., Fiocco, D., Angel Pardo, M., López, P., Spano, G., 2015. Zebrafish gut colonization by mCherry-labelled lactic acid bacteria. Appl. Microbiol. Biotechnol. 99, 3479–3490.

Strayer, D.L., 2012. Eight questions about invasions and ecosystem functioning. Ecol. Lett. 15, 1199–1210.

van Elsas, J.D., Chiurazzi, M., Mallon, C.A., Dana, Elhottovā, Krištůfek Václav, Salles JF., 2012. Microbial diversity determines the invasion of soil by a bacterial pathogen. Proc. Natl. Acad. Sci. 109, 1159–1164.

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89, 670–679.

Zhang, X., Bishop, P., 2001. Spatial distribution of extracellular polymeric substances in biofilms. J. Environ. Eng. 127, 850–856.

Zhao, T., Podtburg, T.C., Zhao, P., Schmidt, B.E., Baker, D.A., Cords, B., et al., 2006. Control of Listeria spp. by competitive-exclusion bacteria in foor drains of a poultry processing plant. Appl. Environ. Microbiol. 72, 3314–3320.

Zumstein, E., Moletta, R., Godon, J.-J., 2000. Examination of two years of community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-strand conformation polymorphism analysis. Environ. Microbiol. 2, 69–78.