The effect of permeate drag force on the development of a biofouling layer on pressure driven-membrane separation system

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Running title: Effect of cross-flow on membrane biofouling.

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Abstract

The effect of permeate flux on the development of a biofouling layer on cross-flow membrane separation was studied by means of a bench scale system consisting of two replicate 100 kDa MWCO-tubular ultrafiltration (UF) membrane modules, one allowing the flow of permeate and the other does not (control). The system was inoculated with *Pseudomonas putida* S-12 tagged with a red fluorescent protein and operated at laminar flow regime under sterile conditions with a constant fed of diluted (1:75) Luria-Bertani medium. Biofilm morphology was studied by means of Field Emission-Scanning Electron Microscope and Confocal Scanning Laser Microscope and subsequently quantified by image analysis, as well as live counts and permeate flux monitoring.

Biofilm development was highly enhanced in the presence of permeate flow, causing the build-up of complex three-dimensional structures along the membrane. Active bacterial transport towards the membrane by permeate drag was found to be more dominant mechanism by which cross-flow filtration contributes to the buildup of a biofouling layer than transport of nutrients. Cellular viability was found to be not essential for transport and adhesion under conditions of cross-flow, since the permeate drag overcomes the effect of bacterial motility.
Introduction

Membrane separation is becoming a wide-spread technology for water and wastewater treatment and purification (35). A membrane is basically a selective porous medium, which allows the transfer of certain molecules to the permeate side based on size and charge, depending on the process type. In cross-flow membrane separation two simultaneous streams are present: the feed stream, flowing in parallel to the membrane walls and the permeate stream, flowing perpendicularly to the membrane surface (Fig. 1).

The movement of particles, colloids and nutrients towards the membrane surface enhances the development of the biofilm layer (biofouling). Besides the substances of biological origin, i.e., cells and EPS (Extracellular polymeric substances), the biofouling layer may include inorganic and organic substances and particulate material rejected by the membrane. Biofilms provide clear advantages to the microorganisms: protection from stress conditions in the environment, better metabolic cooperation and higher densities facilitating genetic exchange (15, 52).

Biofouling can become a significant problem if biofilm growth is not controlled, because it can lead to clogging of the membrane, impairing the ability of the system to function properly (7).

In the case of cross-flow membrane filtration, the net velocity of particles towards the membrane surface is largely determined by normal convection with small contributions by tangential convection and Brownian diffusion (51). Non-specific interfacial forces seem to dominate bulk transport, and thus, govern deposition phenomena. These interfacial forces are caused by interactions such as van der Waals, steric, Lewis acid-base, electrostatic double layer phenomena and hydrodynamic conditions (31). As compared to dead-end filtration, cross-flow filtration has the
intrinsic advantage that the feed stream generates a tangential shear force on the membrane walls, thereby diminishing biofouling.

The initial attachment of bacteria on membranes is largely controlled by physicochemical factors such as solution chemistry, surface properties of the membrane and particles and the hydrodynamic conditions (2, 4, 9, 11, 18, 19, 34, 57). Bacterial factors have also an important role in attachment. Increased production of specific surface proteins and exopolysaccharides is resulted in increased cellular adhesiveness and biofilm formation (28, 49, 53). The expression of these components is mediated by GGDEF domain-containing proteins in different bacteria such as *P. putida*, *Escherichia coli*, *Salmonella enterica*, *Vibrio cholera* and *Pseudomonas aeruginosa* (23, 32). The surface attachment, especially in the early stages of colonization, may also be affected by bacterial motility and particularly by the twitching motility, a form of surface translocation mediated by flagella and type-IV pili (14, 26, 42, 43). Flagella has been proposed to be important in the initial attachment phase of *P. Putida* on plant roots and fungal hyphae (55, 58) but on other surfaces it was not shown to be necessary (22). Moreover, a hyper-flagellated mutant strain showed to be impaired in it's initial biofilm formation (13). Flagella is also known to play a role further in the structural development of the biofilm. Subsequent to the formation of microcolonies, flagellum-driven motility takes a key role in the formation of loose protruding structures (54). The expression of flagella was found to be in strong relation with surface attachment: down regulated quickly after surface attachment and upregulated at later stages of biofilm development (32).

Motility was found to be involved in the bacterial attachment to surfaces in both flowing (33, 39) and static systems (43, 47, 56). In cross-flow regime, there is a trade off between the shear force (acting in the horizontal direction and releases bacteria off
the surface by shearing them off the surface) and the permeation drag force originated by the pressure gradient across the membrane or transmembrane pressure (acting in the vertical direction, carrying bacteria and other particles towards the membrane).

Little is known so far about the importance of motility on bacterial attachment and biofilm development in cross-flow membrane filtration, which represents a different flow field than the conventional flow pattern of unidirectional flow.

The permeation drag force, which acts perpendicularly to the stream lines is not only an additional strong force assisting the cells to penetrate the hydrodynamic layer (18, 19), but may influence the biofilm development, as follows: (i) speeding up and enhancing attachment of the first cell clusters at the early attachment phase, (ii) increasing migration of nutrients and gases towards the base of the biofilm, (iii) improving removal of metabolites and spread of signal molecules. The influence of cross-flow on the physical properties or morphology of biofilms had not been thoroughly explored. Yet, both morphological and physical differences between biofilm grown with or without cross-flow can be expected, due to this added perpendicular flow vector. Biofilm thickness and density are two important parameters which may change in the presence of permeation, due to the enhanced flux of nutrients. Changes in these parameters may introduce diffusional limitations and affect the microenvironment surrounding the cells (46).

The working hypothesis of the present research is that the permeation drag force overcomes the local shear force and shadows the importance of the swarming motility in the adhering bacteria, making the swarming motility irrelevant in this case. Once attached, the bacteria that colonize the membrane surface utilize this nutrient flux into biomass, and form the EPS of the biofilm. The aim of the present study was, therefore, to investigate how cross-flow filtration quantitatively affects structural
biofilm parameters on a bench scale system, consisting of tubular UF membrane modules, inoculated with a fluorescently tagged *Pseudomonas putida* S-12 and operated at laminar flow regime under sterile conditions in continuous mode. Biovolume, thickness, density and coverage area, morphology, as well as the decrease in the permeate flux through the membrane during the build-up of the biofouling layer were determined. The effect of the cross-flow on microbial cells transport and the importance of motility during initial colonization were studied as well. These parameters were obtained by CLSM imaging and subsequent image analysis upon induction of biofilm formation.

**Materials and Methods**

**Bacterial strain**

Wild type *P. putida* S12 (ATCC number: 700801) (27) was used as the model organism for this study. The mini-transposon (plasposon) pTn*Mod*-RKm' (GenBank accession number: AF061930) was used as a suicide delivery vector to mark the *P. putida* S12 chromosome with DsRed (RFP) and a kanamycin resistance gene (*kanR*) (17). A single-species biofilm was chosen in order to simplify the test-system and provide means for easier tracking of the biofilm development, without the influence of cross-relation between different bacterial populations.

**Inoculum and media**

Luria-Bertani (LB) broth was used for generation of the starter cultures (grown for 16 h at 30°C in shaken flasks) used for inoculation of the experimental cultures. Experimental media consisted of LB broth diluted 1:75, unless otherwise indicated. The proper dilution was achieved by proportional feed of water and 1:10 LB medium by means of two peristaltic pumps. Kanamycin (30 µg/ml) was added to the maintenance, inoculum and experimental media as a mean for selection.
The experiments were started by adding a volume of the starter culture (3.3 OD) into the reaction medium (140 ml) to reach an initial cell concentration of approximately $1.4 \times 10^7$ cells/ml in the reactor (Fig. 2). All experiments were performed under sterile conditions.

**Flow-through cell system**

A dual channel tubular flow-through cell system allowing work under sterile conditions was applied (Fig. 2). The flow-through cells (13 cm length-L and 12.5 mm diameter-D) were equipped with a 100 kDa molecular weight cut-off-UF tubular membranes made of PVDF (PF100, PCI). Unless otherwise stated, one cell was run under cross-flow (+CF), allowing the passage of permeate through the membrane, and the other was run without cross-flow (-CF) in a unidirectional flow (Fig. 2). A concentrated nutrient feed solution was directly diluted with double distilled water (DDW) to the desired concentration in a 140 ml reactor by regulating the flow of two parallel peristaltic pumps (Cole-Parmer, USA). Cells, reactor and reservoirs were all of Pyrex glass. The different components of the system were connected with silicone tubing and recirculation was done by means of a peristaltic pump. The system was equipped with pressure gauges and rotameters.

Biofilms of *P. putida* were allowed to develop on the membrane surface, as a model for biofouling layer development, under sterile conditions. In all cases all the components of the system, except the membranes and the pressure gages, were fully autoclaved (121°C for 20 min) prior to each experiment. After the assembling of the system, a 0.5% w/w NaOCl solution was run in the system for at least 3 hours and thereafter the system was thoroughly rinsed with sterile DDW for 16 hours. In order to check for sterility, plating of the rinsing water was done on LB agar plates prior to each experiment.
After inoculation the influent flow rate was maintained at 3.5 ml/min, giving a dilution rate of 0.025 h\(^{-1}\) (corresponding to a hydraulic residence time of 40 min). This rate was above that of washout rate for \(P.\ putida\) S12 in the diluted medium, to minimize suspended growth and to encourage biofilm growth (48). Effluent samples were taken right after inoculation and were found to be in the range of \(10^7\) cells/ml. The average flow velocity (\(\bar{u}\)) was maintained at approx. 0.065 m/s and the corresponding calculated Reynolds number was 910 (at 30\(^\circ\)C), i.e., laminar flow. The shear rate on the membrane surface (\(\Gamma_w\)) in laminar flow regime was calculated as \(\Gamma_w=8\bar{u}/D=41.6\) sec\(^{-1}\).

Permeability (\(L_p\)) is defined as the specific permeate flux (\(J\)) through the membrane relative to the transmembrane pressure drop (\(\Delta P\)) across the membrane, according to \(L_p=J/\Delta P\) (l/m\(^2\).h-bar), neglecting osmotic pressure changes. Permeability was calculated at different time points in order to track the kinetic pattern of the biofouling layer build-up depicting the blockage of the membrane. The transmembrane pressure was calculated as \(\Delta P=(P_{\text{in}}+P_{\text{out}})/2\), neglecting the pressure on side of the permeate which was discharged at atmospheric pressure, whereas \(P_{\text{in}}\) and \(P_{\text{out}}\) are the pressure of the feed and retentate streams, respectively (see Fig. 2). Unless otherwise stated, the experiments were conducted at a fixed initial \(\Delta P\) of 1 bar.

A sampling port was positioned on the overflow line at the exit of the reactor.

Autoclave-sterilized 0.22 \(\mu\)m air filters (Millipore Millex-FG50) were positioned on the reactor and the feeding carboys, to allow free aeration of the system.

**Comparative growth of biofilms on membranes with/without cross-flow**

Independent experiments were run for different time-periods (1 h up to 6 days), each consisting of side-by-side +CF and –CF membrane modules. The system was pre-run with a nutrients solution for 4 h in filtration mode, i.e., with permeate flux, prior to
inoculation (time zero). After inoculation, as described above, a sample of the liquid in the system was collected and plated for live cells count. Permeate flow rate from the +CF membrane was recorded during the experiments at different time points, as indicated.

At the end of the experiments, the membranes were taken off the system and were thoroughly washed with sterile saline solution to discard all loosely attached bacteria. To count the attached bacteria, two pieces of 1 cm long membrane (giving an area of 3.93 cm² each) from each membrane were vortexed in a glass tube for 40 seconds with 4.5 ml of saline+0.1% Tween-80 solution, containing 2.5 g glass beads (D=3 mm). A serial dilution was prepared and plated on LB agar plates. The plates were incubated at 30°C for 16 h. To analyze the biofilm structure, membrane samples were sliced and prepared for microscopy analyses as described below.

**Effect of the permeate drag force on bacterial transport and biofilm formation**

For these experiments, the flow-trough system similar to that described above but including three parallel channels was applied. The system was pre-run with nutrients solution for 4 h and aseptically inoculated (time zero), as described above. After inoculation a sample of the liquid in the system was collected and plated. Then, all three modules were operated as +CF membranes for 15 minutes, allowing the flux of permeate through the membrane, in order to allow bacteria to attach equivalently to all the three membranes in full recycle mode (batch mode). During this phase no fresh nutrients feed was introduced to the system in order to keep the bacterial concentration in the system constant. Thereafter the system was aseptically evacuated and then refilled with fresh nutrients feed (purging phase). This procedure was repeated at least 5 times for one hour, in order to remove the maximum of planktonic cells possible. At the end of this phase, a sample of fluid from the system was
collected and plated, to track the concentration of suspended bacteria left in the
system. Concomitantly, one module was disassembled from the system and the
membrane in it was thoroughly washed with sterile saline solution to discard loosely
attached bacteria, as mentioned above, and plated to quantify the attached bacteria.
From this point onwards, the nutrients feeding rate was increased to 1.5 l/h (retention
time equals to 8 min), in order to wash out all possible remaining planktonic bacteria.
The two modules left were run one as +CF and the other as –CF, as described
previously, for an additional 4 hours. At the end of this running period a sample of the
liquid was collected and plated, in order to track the suspended cells concentration in
the system, which was found to be in the range of 10^4 cells/ml, three orders of
magnitude smaller than right after inoculation. Then the two modules were
disassembled and the membranes were washed and plated as mentioned above.

The influence of bacterial viability on the adherence to the membranes

Two separate flow-through cell systems were run in parallel under sterile conditions.
One flow-through system was a dual-module similar to that described in Fig. 2,
inoculated with dead cells (described below) and run one +CF membrane and one –
CF. The second flow-through system was a single-module system, inoculated only
with live cells +CF. Both systems were inoculated to the same final cells
concentration. In this set-up, both systems were operated by a three-headed peristaltic
pump (Cole-Parmer) in flow conditions same as above. Running time was set as 1
hour, to allow early attachment while minimizing duplication of the live bacteria.
After inoculation, a sample of the liquid in the system was collected and plated. Both
systems were run with full recycle (batch mode) in order to avoid differential dilution
and washout.
At the end of the running time the membranes were disassembled and thoroughly washed with sterile saline solution. Membrane specimens were cut, thoroughly washed and directly observed by fluorescent microscope for analysis of coverage area.

For preparation of live and dead RFP-tagged *P. putida* S12-cell suspensions, bacteria were grown and harvested according to Solomon and Matthews (50). Briefly, cells were grown overnight in LB, then split in two. Cells had been killed with 2.5% glutaraldehyde. Microscopic observation showed that the dead cells retained their fluorescence properties.

**Microscopy**

*Confocal laser scanning microscopy (CLSM).* Biofilm development was visualized by using a MRC 1024 CLSM (Bio-Rad, Hemstead, UK) equipped with a Nikon Plan Apo 63x1.40 objective and by using a LSM Zeiss 510 Meta equipped with a Zeiss AxioImager Z1, equipped with detectors, lenses and filter sets for monitoring the fluorescent staining applied. The EPS was visualized by using Concaavalin A (FITC) staining (40, 41). Bacterial cells were visualized by the RFP tagging. Staining with nucleic acid stains Syto9 (Invitrogen-Molecular Probes, S34854) and Propidium iodide (PI) (Sigma, P4170), for visualization of total and dead cells, respectively, was done according to the manufacturers instructions.

Parameters of the CLSM were set once and applied evenly as much as possible for the rest of the experiments, in order to ensure quantitative comparison of the results. Wavelengths were set according to manufacture instructions (Concaavalin A-FITC and Syto9: ex. 488 nm, em. 498; RFP: ex. 563, em. 580 nm, PI: ex. 493; em. 630).

*_fluorescent Microscopy._ Early stages of biofilm development were tracked by observation of fluorescently tagged bacteria, using a Leica Dmire2 inverted
microscope and Zeiss Axio-observer 200M inverted microscope. Z-cross sections were acquired by using x40 and x63 lenses (Z-cross sectioning was needed due to the curved nature of the membrane surface).

Scanning Electron Microscopy (SEM). Membrane samples for SEM were fixed with glutaraldehyde and dehydrated in an ethanol gradient in the cold, as described elsewhere (3, 30). Hexamethyldisilazane (HMDS) was used to dry the cells instead of the critical point drying. This method was reported to be better suitable for drying samples of cells for SEM examination without causing cell structure disruption (3). Samples were sputter-coated with carbon and were visualized by a Leo Gemini 982 High Resolution SEM. All chemicals employed were of electron microscopy quality (Sigma Chemical, St. Louis, MO).

Image analysis. PHLIP software (http://phlip.sourceforge.net) (38) is an open-source software which was specifically designed for 3D biofilm analysis. PHLIP automatically sets the threshold value using the Otsu algorithm (44) and calculates the architectural parameters. PHLIP runs on Matlab as a platform and requires an additional definitions file, which was automatically produced by Auto-PHLIP-ML software (http://sourceforge.net/projects/auto-phlip-ml) (37). Auto-PHLIP-ML also removes bias from biologically insignificant pixels by removing extraneous images. In this work PHLIP calculations were applied to determine biovolume, substratum coverage, area to volume ratio and mean thickness. Biovolume is the volume of the biomass, as measured by accumulation of foreground pixels which are attributed to biomass (by external fluorescent staining or inherent bacterial red fluorescent protein). The threshold was set automatically by PHLIP (by running the Otsu algorithm), and manually verified and adjusted when necessary (38).
PHLIP was run in "no connected volume filtration" (CVF) mode. CVF option removes pixels that are not connected to the substrate through the connection to other neighboring pixels (38). In our study CVF was found to cause bias in the results, due to the curved character of the membrane surface. Data was exported in an XML format and statistically analyzed using Excel.

ImageJ (http://rsb.info.nih.gov/ij/) was used for 3D imaging (using the plugin "Volume Viewer 1.31"; http://rsb.info.nih.gov/ij/plugins/volume-viewer.html (6)) and for analysis of coverage area at the early development stages (monolayer) of the biofilm.

Z-stack images acquired by fluorescent microscopy were processed and focused by "Extended depth of field" plugin (http://bigwww.epfl.ch/demo/edf/#soft, (21)) to produce a focused merged image of the whole scanned area, for subsequent coverage area analysis.

**Results**

**Effect of cross-flow on build-up, morphology and structural parameters of biofilms developed on membranes**

The purpose of these experiments was to perform a comparative study of biofilm development on membranes, with and without cross-flow filtration. The -CF membranes were depleted of a pressure gradient across them, thereby preventing permeate flux as well as the accumulation of a concentration-polarization layer. In the +CF a pressure difference across the membrane was created and consequently permeate flux was allowed, thereby generating a convective flow towards the membrane surface.

The bacterial adherence and build-up of the biofilm layer on the +CF membrane caused a typical exponential decrease of the membrane permeability, resulting in a
decline of ~75% after 48 h (Fig. 3). Following this time, the permeability profile reached an asymptotic value, which corresponded to the fully developed biofilm (namely, reached a saturation resistance under these conditions). We predicted that permeability decline would take place short after the inoculation. Measurement of the permeate flux in several independent replicate experiments showed that a 22% permeability decline already takes place within the first hour once bacterial deposition and biofilm formation took place, becoming moderate thereafter (see Fig. 3).

The biofilm build-up was more evident in the +CF membranes as depicted by a constantly higher thickness developed than biofilm grown on the –CF membranes (Table 1). Within 12 h of the inoculation period, the biofilm on the +CF membrane consisted of a thin layer of cells (varying from monolayer up to 8 µm) while the –CF membrane consisted of a sparse layer of cells which randomly colonized the membrane surface. This difference between the biofilms on the +CF and –CF membranes persisted also at 20 h, although both biofilms became denser. At 6 days and onwards, the +CF membrane became clogged by the biofilm layer, the permeate flux decreased to negligible levels (Fig. 3), and the hydraulic conditions in the membrane approached a –CF performance (in terms of cross-flow performance).

During this time, the cells attached to the –CF membrane developed a complex 3D structure, and its thickness grew accordingly, yet, not as thick as the +CF membrane. The variability in the thickness of –CF membrane after 6 days was significantly higher than the +CF membrane, as evidenced by the standard deviation (Table 1). This is in correlation with a higher frequency of uncovered patches in the -CF membrane compared to higher homogeneity of coverage in the +CF ones.

The morphology of the mature biofilms, as evidenced by CLSM and HR-SEM observations, displayed a complex and filamentous 3D structure, including cave-like
structures and tunnels, presumably allowing the transfer of nutrients and gases towards the base of the biofilm (Fig. 4). This structure is common in biofilms developing in fast-flowing environments, in line with previous reports (52).

The bacterial density of the biofilms accumulated on the membranes was measured by plate cells counts, normalized to either the membrane surface area (Fig. 5) or to the biovolume (Table 1). The bacterial density (relative to the surface area of coverage) of both +CF and –CF increased as the biofilm developed, as expected. However, a difference of more than 5-orders of magnitude between –CF and +CF membranes was seen at the early attachment stage, namely after 1 hour post-inoculation (Fig. 5). This difference gradually decreased with time and after 12 h the +CF biofilm was 3-orders of magnitude denser than –CF biofilm and 1.5 orders after 6 days. This decrease correlates the convergence with time of both systems to a –CF like-regime and expresses a saturation level for colonizing bacteria and reproduction/detachment of the anchored bacteria at the hydrodynamic conditions applied. As stated before, the reason for the definitively higher concentration of cells on the +CF membrane is explained by the convection force of the permeate stream, which actively transported bacteria towards the membrane surface.

The increase in biofilm density was also apparent when looking at the increase of bacterial density normalized to biovolume. The biovolume increased to a lower extent than cells count, apparently limited to certain saturation value in correspondence to the hydrodynamic conditions applied, namely the shear rate. As a consequence of this, the biofilm became denser as it developed.

In addition, a gradual change in the relative biofilm composition that took place during its development might contribute as well to the change in the increase of the bacterial density determined. Indeed, this is depicted by the biovolume of the bacterial
biomass relative to the biovolume of the EPS in the biofilm, which changed with time as the biofilm became denser (Table 1). For the +CF membrane, at the early stages (12 and 20 h) the same amount of EPS and bacteria was noticeable (ratio=1:1). After 6 days this ratio increased in favor of the bacterial biomass, as the biofilm became denser.

For the –CF membrane, after 12 h, a low bacterial density layer instead of a defined biofilm was formed and therefore it was taken into account for the biovolume ratio calculation. After 20 h the bacterial density increased and the ratio became close to the +CF membrane. After 6 days the –CF biofilm displayed a developed structure, in which most of the biovolume corresponded to bacterial biovolume. Whilst, the +CF biofilm which was denser than the –CF one, had a 2-fold higher bacterial biovolume to EPS biovolume ratio than the –CF biofilm.

The relative location and abundance of the different components of the biofilm (such as EPS, protein and bacteria) is also of interest, since it determines the physical stability and tolerance of biofilms to different stress conditions. Differential CLSM observations and subsequent image analysis showed that the mature biofilm, i.e, 6-days old, was surrounded by EPS of 2-3 µm thick, whereas the bottom parts were composed mostly of bacterial biomass (Fig. 6). The EPS covering layer was reported to serve as a protecting layer (20, 52). At the early stage of development, i.e, 20-hours old, about half of the biovolume was composed of EPS, mostly located towards the external surface of the biofilm (Fig. 7), while the internal parts were composed mostly of bacterial biovolume. This phenomena was characteristic for both the +CF and –CF membranes, according to the rate of development.

**Importance of the permeate drag force as a mean of bacterial transport and nutrients flux towards the membrane surface**
The permeate drag force augments the transfer of bacteria towards the membrane surface was well as the flow of nutrients and adsorbed gasses through the membrane surface, providing the attached bacteria with constant supply of nutrients and oxygen. These two processes occur simultaneously when cross-flow filtration is present. The purpose of this part of the work was to evaluate the contribution of each of these two processes to the build-up of the biofilm layer.

In order to evaluate the cross-flow permeate drag force as a mean of bacterial transport the system was inoculated and operated for one hour with and without cross-flow. This time is long enough to allow the bacteria to attach to the membranes but sufficiently short to avoid any noteworthy reproduction of the adhered bacteria. Plate count measurements showed a difference of 5-orders of magnitude between both membranes, 1.3 x10⁶ cells/cm² on the +CF and <12 cells/cm² on the –CF. This difference in the adhered cells density was also evidenced by microscopic observations (Fig. 8).

In order to evaluate the importance of the cross-flow permeate force in supplying a flux of nutrients and dissolved gases, an experiment was performed in which three replicate membranes were operated as +CF for the first 15 min after inoculation, allowing identical passage of permeate. Then the system was thoroughly washed from planktonic bacteria and one membrane was taken out for plate counting (control for initial colonization). The two remaining membranes were then operated as +CF and -CF membranes for additional 4 h. This setup allowed a similar initial bacterial colonization of the membranes during which the permeate stream served as a mean of nutrients and gasses supply. Plate counts of the +CF membrane was 13.0±1.1x10⁶ cells/cm², which was 5 times higher than the –CF membrane (2.30±0.09x10⁶) and 12 times higher than the control membrane (1.10±0.01x10⁶). As can be seen by
comparing the −CF and the control membranes, a net cells growth equivalent to one doubling took place at the membrane surface, i.e., potential biofilm growth. However, when comparing the +CF and the control membranes, the bacteria replicated approximately 12-fold. Hence, the contribution of the permeation as a mean of nutrients supply to the bacteria colonizing the membrane is one-half order of magnitude for a 4 h time-period, resulting in a ratio of more than 1:6 in favor of the permeation as a mean of bacterial transport towards the membrane surface.

In conclusion, these results clearly indicate that the cross-flow convective force enhances bacterial transport (increase of 5 orders of magnitude in bacterial density on the membrane within 1 h), while increase in bacterial density due to transport of nutrients to the biofilm caused an increase of only half an order of magnitude after 4 h.

Adherence of live bacteria to the membrane surface under cross-flow conditions

This part of the study was aimed to evaluate quantitatively the contribution of the early attachment of living bacteria onto the membrane surface under cross-flow conditions. For this purpose, the adhesion of live and dead bacteria after 1 h was compared. Surface coverage analysis shows that +CF membranes were significantly covered to an almost identical extent, regardless of cell viability (live bacteria: 14.0±5.9% of the area was covered with bacteria; dead bacteria: 19.6±12.7%), while −CF were covered to a much lesser extent (0.5±0.7% for dead cells and similar results for live cells, as measured in other experiments). Dead cells even appear to cover more area than the live bacteria in two different experiments (Fig. 9), but this difference was not statistically significant. This difference can be explained by changes in the net charge and surface properties of the glutaraldehyde-inactivated cells upon interaction with the membrane surface than live bacteria (10, 29). In the
case of -CF membranes a very low dead-cell adherence, as evidenced by the low
coverage area was found for dead cells (Fig. 9C) and for live cells (see Fig. 8).
Overall, these findings clearly indicate that the convective force driven by the cross-flow is the dominant factor responsible for bacterial cell (and other similar colloids) transport to the membrane.

Discussion
Cross-flow is an important process in water and wastewater membrane separation systems in which the main stream (feed) flows in parallel to the membrane walls, while the permeate stream flows perpendicularly to the feed stream, towards the membrane wall. A major operational problem in this process is the build-up of a biofouling layer, which blocks the membrane and causes significant energy losses. In order to study the contribution of the permeation drag force in the creation of a biofouling layer, a tubular flow-through cell system, mimicking simple pipe-flow conditions, with and without permeate flow was operated. We found that membranes operating in cross-flow regime were colonized very rapidly, already causing a reduction of permeate flux of approx. 22% within the first hour. These findings are in line with previous observations of membrane colonization which occurred within a matter of minutes (34). In the absence of permeation (i.e, only parallel flow), the deposition of bacteria was slower since the planktonic bacteria had to overcome the parallel drag forces to reach the surface, as in the generic case of biofilm initiation in flowing systems, and consequently biofilm developed slowly. Similarly, Kang et al (31) reported that even in the absence of permeate stream there is deposition of bacteria on the membrane.

Structural parameters characterizing the biofilm layer, such as thickness, biovolume and bacterial density, increased gradually as the biofilm developed. Interestingly, the
differences in these parameters between the +CF and –CF membranes gradually decreased with running time, converging to an almost similar magnitude after 6 days of operation. This convergent time-profile can be explained by two processes occurring in parallel: (i) as planktonic cells attached to the membrane, settled and started to form biofilm (~12-20 h) the +CF membrane became clogged and the permeate flux decreased in an exponential pattern, resulting in lower permeate drag force that transports bacteria and dissolved nutrients to the membrane surface; (ii) the –CF membrane slowly developed in a conventional biofilm pattern under diffusive conditions. Moreover, the hydraulic conditions applied (retention time < doubling time of planktonic cells) favored proliferation of attached vs. planktonic bacteria, whose concentration in the system consequently decreased. These events gradually reduced the influence of convection forces towards the membrane surface due to permeate flux.

Our findings further show that transport of bacteria to the membrane surface (by convection force) is the main cause of biofilm enhancement, while the flux of nutrients to the biofilm is a less important mechanism. This effect is most significant at the early stages of the biofilm development, when the active transport of planktonic cells towards the membrane surface is at its maximum and the dependency of the attached bacteria (still at a monolayer stage) on a nutrients flux is less crucial. Once the biofilm has been fully developed, the dominant contribution of the permeate stream is by nutrients supply, even though at this stage the permeate flux is significantly lower than at the early stages. Therefore, both effects of permeation are important to biofilm development, but their relative magnitude differs at the different phases of development.
Twitching and swarming motility were found to be important for cell aggregation (25, 43) and hence the creation of the biofouling, especially in the early stages (12, 55). Therefore, dead cells are not supposed to be able to perform active adherence actions, but to be all dependent on adsorption due to chemical interaction forces (31) and the presence (or absence) of permeation drag force. *P. putida* exhibits surface motility similar to swarming at room temperature (18-28°C), the temperature applied in this research. At this temperature (but not at 30°C) the bacteria produce type four pili and polar flagellum. *P. putida* exhibits flagella independent surface movement, but the pili and LPS-O antigen are required for surface movement (36). Although these results were obtained for *P. putida* KT2440, the only fully sequenced *P. putida* strain, they may be applied for *P. putida* S12. Indeed, microarray hybridizations showed that S12 exhibits the highest genomic similarity to KT2440 (5). Our results indicate that in the case of cross-flow filtration, the permeation drag force overcomes the need for bacterial motility, and therefore dead cells will reach the membrane surface in an equal amount to the live cells. This suggests that under the influence of the permeation drag force, self-motility is not essential for primary colonization.

A study on four strains of bacteria that produce biofilms on RO membranes suggested that in the presence of permeation, convective permeate flow reduces the dependency of bacterial cells on flagella-mediated swimming motility in establishing the initial cell-to-surface contact (45). Our findings support this hypothesis. In a static system, flagellar motility is important for *P. aeruginosa* surface attachment, whereas type IV pili facilitate microcolony formation (43). However, type IV pili and flagellar motility do not significantly affect biofilm formation in a constantly flowing system (16). This suggests that cells subjected to shear force, have limited surface movement via type IV twitching motility and the initial microcolony formation is affected mostly by
other mechanisms, primarily cellular division rather than a combination of cell 
clustering and division. In conclusion, our findings indicate that in membrane separation systems containing 
considerable loads of microorganisms (such as in the case of sea water desalination 
plants and wastewater treatment plants, in which bacterial concentrations of $10^5$-$10^6$ 
CFU/ml are present), the convective transport of bacteria to the membrane remains 
the main cause for biofilm formation. Furthermore, our results suggest that 
bacteriostatic pretreatment of the feedwater to reduce bacterial colonization may not 
be very helpful in membrane facilities, since the dead cells will deposit on the 
membrane wall and cause clogging. That is, efficient removal of feed microorganisms 
by physicochemical and/or physical separation should be performed. Yet, limiting 
nutrients availability in the feed seems to remain the most effective countermeasure 
for biofouling control as it represents potential biomass.
Acknowledgments

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List of figures

Figure 1. Schematic representation of cross flow ("+CF") vs. non cross-flow filtration ("-CF"). Under cross-flow filtration there is a permeate stream which acts perpendicularly to the feed stream, whereas under no cross-flow regime there is a unidirectional flow.

Figure 2. Schematic diagram of the dual channel tubular flow-through cell system. Cells were 13 cm length by 12.5 mm diameter. A concentrated nutrient fed solution was directly diluted with distilled water to the desired concentration in a 140 ml reactor by regulating the flow of the feed and dilution peristaltic pumps. Pressure gages and rotameters were used for continuous determination membrane permeability. The system was run under sterile conditions. Membrane modules can be seen on the upper-left corner (Flow-through cells). Where indicated, a third membrane reactor was added.

Figure 3. Typical time-profile of the relative permeability of a +CF membrane due to the build-up of the biofouling layer. The asymptotic value of the permeate flux corresponds to the fully developed biofilm (biofouling) layer. Initial permeability was 0.6±0.06 l/m².h-bar.

Figure 4. FE-SEM micrographs of 20 and 144 h-old biofilm, grown under cross-flow and non-cross-flow conditions. After 20 h, noticeable differences in the coverage area and biofilm development can be easily seen. After 144 h, both biofilms had developed to a complex 3D structure. However, in the –CF membrane not fully covered areas could still be seen.

Figure 5. Bacterial density of the biofilm at different times, measured as live-count per membrane area. Values given represent the average of at least 4 independent measurements, obtained from four independent experiments, performed as a set of
two parallel runs each time. Bars represent SD. Bacterial density of –CF at 1 and 12 h represent an estimate only, since their values were lower than detection limit.

**Figure 6.** Biofilm structure. (A) Typical CLSM micrograph of a 144 h-old –CF biofilm. The EPS covers the bacteria on top of the bacterial structure. Maximal intensity merge of all the Z-stack; (B) 3D image of the biofilm structure; (C) Relative abundance of the EPS and bacteria as measured by coverage area at different Z-depths. The green line represents EPS and the red color represents bacteria. Total thickness: 22 μm (1 μm per cross-section). Note that on the first three cross-sections EPS are more abundant while all the deeper cross-sections bacterial biovolume is dominant.

**Figure 7.** Typical image of a 20 h-old +CF biofilm. (A) Maximal intensity merge of all the Z-stack; (B) 3D imaging of the biofilm structure, cut in the middle of the biofilm; (C) Relative abundance of the EPS and bacteria as measured by coverage area at different Z-depths. The green line represents EPS and the red color represents bacterial biovolume. Total thickness: 13 μm (1 μm per cross-section). Note that on the first nine cross-sections the EPS is more abundant while in all the deeper cross-sections bacterial biovolume is predominant.

**Figure 8.** SEM micrographs of a -CF membrane (A) and +CF membrane (B), after 1 hour of running time. The +CF membrane is populated with bacteria adhered to the surface, while the –CF is clean of adhered bacteria. Scale bar=20 μm.

**Figure 9.** CLSM micrographs of live (A) and dead (B) cells deposited under cross-flow filtration and dead cells under non-cross-flow filtration (C). Note the notably lower number of bacteria attached without cross-flow. Live cells grown under non-cross-flow filtration displayed a similar picture.
Figure 1

Parallel flow

Perpendicular flow

Cross-flow

Membrane
Figure 2

[Diagram showing flow-through cells, recycling pump, permeate, reactor, dilution pump, feed pump, water, nutrients + antibiotic, flowmeters, and overflow.]
Figure 3
Figure 4

- Cross-flow

+ Cross-flow

20 h old

144 h old
Figure 5

Bacterial density (log CFU/cm²)

Time (h)

- CF
+ CF

0 2 4 6 8 10

0 12 20 144
Figure 6
Table 1. Biofilm properties

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Thickness(^a)</th>
<th>Bacterial density(^b)</th>
<th>Bacterial/EPS biovolume ratio(^c)</th>
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<td>+CF</td>
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\(^a\)Biofilm thickness (µm), measured by CLSM. Values represent mean ±standard deviation of 15 replicates.

\(^b\)Values are given as CFU normalized to biovolume unit (µm\(^3\)) as a ratio of at least two independent experiments, measured at least ten times each. Standard deviations and Ttest could not be computed since bacterial cell counts were done independently of the biovolume measurements.

\(^c\)Values are gives as a ratio of bacterial biovolume (µm\(^3\)) to EPS biovolume (µm\(^3\)).

\(^d\)N.D.: Not detectable.