A bilayer coarse-fine infiltration system minimizes bioclogging:
The relevance of depth dynamics

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Abstract

Bioclogging is a main concern in infiltration systems as it may significantly shorten the service life of these low-technology water treatment methods. In porous media, biofilms grow to clog partially or totally the pore network. Dynamics of biofilm accumulation (e.g., by attachment, detachment, advective transport in depth) and their impact on both surface and deep bioclogging are still not yet fully understood. To address this concern, a 104 day-long outdoor infiltration experiment in sand tanks was performed, using secondary treated wastewater and two grain size distributions (GSDs): a monolayer system filled with fine sand, and a bilayer one composed by a layer of coarse sand placed on top of a layer of fine sand. Biofilm dynamics as a function of GSD and depth were studied through cross-correlations and multivariate statistical analyses using different parameters from biofilm biomass and activity indices, plus hydraulic parameters measured at different depths. Bioclogging (both surface and deep) was found more significant in the monolayer fine system than in the bilayer coarse-fine one, possibly due to an early low-cohesive biofilm formation in the former, driven by lower porosity and lower fluxes; under such conditions biomass is favorably detached from the top layer, transported and accumulated in depth, so that new biomass might colonize the surface. On the other hand, in the bilayer system, fluxes are highest, and the biofilm is still in a growing phase, with high microbial activity and low biofilm detachment capability from the top sand layer, resulting in low surface and deep bioclogging. Overall, the bilayer coarse-fine system allows infiltrating higher volume of water per unit of surface area than the monolayer fine one, minimizing surface and deep bioclogging, and thus increasing the longevity and efficiency of infiltration systems.
1. Introduction

Infiltration systems are low-tech and low-cost water treatment technologies where the quality of the infiltrated water improves progressively during the infiltration path through the porous medium as a consequence of biological, chemical and physical processes (Dillon et al. 2008; Miller et al. 2009). Infiltration basins are used worldwide for groundwater recharge, wastewater treatment or storm water disposal or reclaim and for setting up hydraulic barriers against the intrusion of undesired (polluted or salinized) water to aquifers (Bardin et al. 2002). Furthermore, they are suitable for the treatment of decentralized sewage (Duan et al. 2015) and have been increasingly used as a way of solving water supply stress problems in urban areas (Camprovin et al. 2017) or reducing the degradation of stream water quality (Türkmen et al. 2008). Simplicity, low capital and low operating costs are the main advantages of infiltration systems compared to more technologically sophisticated water treatment methods (Campos et al. 2002).

Successful performance of infiltration systems relies on the development of biofilm (growth and spatial distribution) within the porous media. Biofilms are recognized to be the dominant mode of bacterial life (Barai et al. 2016). Natural biofilms are mixtures of
autotrophic/heterotrophic assemblages (Gette-Bouvarot et al. 2014), composed by algae (microphytobenthos), bacteria, fungi, and protozoa embedded in a matrix of extracellular polymeric substances (EPS) and attached to solid surfaces (Lock et al. 1984). EPS constitute a fibrous gel-type matrix which can be broken down into different organic molecules such as polysaccharides, proteins, lipids and nucleic acids (Flemming and Wingender, 2010; Stoodley et al. 2002) and play a crucial role in the initial attachment of cells to solid surfaces, cohesiveness, biofilm thickness (Flemming and Wingender, 2010) and biofilm resistance to external stressors (Flemming et al. 2016).

Biofilms play a key role on a number of biogeochemical processes (Mermillod-Blondin et al. 2005; Battin et al. 2016) through uptake, storage, and mineralization of dissolved organic matter, as well as assimilation of inorganic nutrients (Findlay et al. 2003). However, an excessive build-up of microbial biomass -cells and EPS- can cause fouling of the porous media, known as bioclogging (Oberdorfer and Peterson, 1985; Baveye et al. 1998; Thullner, 2010; Zhong and Wu, 2013; Brangari et al. 2018). Bioclogging reduces the total volume and the connectivity of the pores accessible to flow (Or et al. 2007), triggering deleterious consequences on infiltration systems: reducing infiltration efficiency and increasing operating costs (Xia et al. 2014). This limits the availability of oxidants and nutrients to reach the microorganisms (Thullner et al. 2002), affecting the quality of infiltrated waters (Dechesne et al. 2004), creating a highly polluted filter at the top layer (Segismundo et al. 2017), increasing the need for frequent rehabilitation (Bouwer and Rice, 1989), and resulting in untimely system failures (Jeong et al. 2018). In short, bioclogging may significantly shorten the service life of infiltration systems (Duan et al. 2015, Hua et al. 2014).

Several works have studied bioclogging processes focusing on the spatial and temporal evolution of bacteria and/or EPS concentrations (Xia et al. 2016; Dupin et al. 2001;
In outdoor infiltration systems, where biofilms include phototrophs and heterotrophs, the contribution of algae in bioclogging might be also relevant (Gette-Bouvarot et al. 2014), since main producers of EPS are both bacteria and algae (Malarkey et al. 2015; Hirst et al. 2003).

Bioclogging is a complex process (Ding et al. 2018; Zhou et al. 2018; Fu et al. 2013) that includes several biofilm stages: attachment, adhesion, maturation and detachment (Hall-Stoodley et al. 2004). In these stages, the combination of biological parameters and hydraulic stresses, such as unit flow and shear forces, determine bioclogging development by regulating the thickness of the superficial biofilm (Gutiérrez et al. 2018) and the biomass accumulation and removal rate (Kim et al. 2010). Another parameter that plays a key role on bioclogging is the grain size distribution (GSD) of the porous media as it shapes the structure, size distribution and connectivity of the pore network, controlling the functions of the porous media-associated biofilm (Santmire and Leff, 2007). On one hand, GSD defines the surface area available for colonization per unit of sand mass, which increases with decreasing average grain size (Mendoza-Lera et al. 2017). On the other hand, GSD controls hydraulic conductivity (Battin, 2000), largest for coarse sands, allowing large supply of solutes in depth (Higashino, 2013) thus influencing biomass establishment and biofilm activity along the vertical profile (Perujo et al. 2017). As stated by Ding et al. (2018), high biological activity could decrease organic matter accumulation (and the occurrence of bioclogging), but on the other hand it may also enhance biofilm growth which can promote bioclogging.

According to de Matos et al. (2018), bioclogging is still a large “black box”, as the main factors that modulate it are not yet well understood.

Two strategies commonly used to control bioclogging are restoration and prevention. The former is based on restoring hydraulic conductivity by replacing the porous media
(Nivala et al. 2012) which can be costly, difficult to implement, and not very effective (Tang et al. 2018). In fact, surface clogging (or “cake-clogging”) may be reversible (e.g., by scrapping), but this is typically not the case for deep clogging (Pholkern et al. 2015). It is therefore necessary to increase our knowledge on bioclogging in order to improve the design and maximize the performance of infiltration media (Farah et al. 2016; Duan et al. 2015). In fact, detachment of superficial biofilm and further transport in depth, as well as the growth and attachment of new biomass in depth play a key role in the process of deep bioclogging in infiltration systems. Furthermore, in spite of its importance, few studies have addressed the occurrence of deep clogging (e.g., Gutiérrez et al. 2018; Kia et al. 2018; Segismundo et al. 2017); further, they focused only on physical clogging but not on biological clogging.

In this paper, we investigated biofilm growth, detachment and vertical-transport linked to superficial and deep porous media bioclogging by means of statistical correlations and multivariate analysis. In the present study, physical clogging was neglected as infiltrated water used was secondary treated wastewater with low suspended solids concentrations. Specifically, the main objectives of this study are (1) to infer how GSD influence biofilm dynamics (biomass attachment, detachment and interstitial transport) in depth; (2) to improve our ability to control bioclogging by understanding how biofilm dynamics modulate the occurrence of surface and deep clogging as a function of the GSDs, and (3) to disentangle the relationship between biofilm activity and biomass accumulation in the occurrence of bioclogging in infiltration systems. To achieve these objectives, we have compared (i) a system composed by a layer of coarse sand placed on top of another of fine sand, termed bilayer coarse-fine system -as in a previous study Perujo et al. (2017) demonstrated that this setup could result in a hot-spot of biological activity--; and (ii) a system composed by a single layer of fine sand. A complementary
specific objective is to evaluate if the former setup could reduce bioclogging development in infiltration systems.

2. Methodology

2.1. Experimental design

The outdoor infiltration experiment consisted in flow-through sand tanks (0.21 m$^3$ of capacity) filled following two different setups in terms of grain size distribution: (i) a bilayer system (denoted CF, standing for Coarse-Fine), consisting of a 20 cm layer of coarse sand (0.9 – 1.2 mm) placed on top of a 20 cm layer of fine sand (0.075 – 0.250 mm), in triplicate; and (ii) a monolayer system (denoted F, for Fine), consisting of a 40 cm layer of fine sand (0.075 – 0.250 mm), in triplicate (Fig. 1). Sand porosity ($\phi$) was 0.4 for the coarse sand layers and 0.32 for the fine ones.

Figure 1 Scheme of the experimental design with two setups (in triplicate) in terms of grain size distribution: (left) monolayer system (F, Fine), with a 40 cm layer of fine sand; (right) bilayer system (CF, Coarse-Fine), 20 cm layer of coarse sand + 20 cm layer of fine sand.

In the upper part of each tank, a valve ensured a constant level of secondary treated wastewater supplied from the Girona urban WWTP (Spain), creating an overlying layer of water and a continuous infiltration resembling vertical flow conditions. A gravel
layer was placed at the bottom of each tank to facilitate water drainage towards the outlet. The experiment lasted 104 days (April to July 2016) with a mean outdoor temperature of 21.6 °C (AEMET – State Agency of Meteorology, Spanish Government) and 234 mm of accumulated rainfall (METEOCAT – Meteorological Service, Government of Catalunya). Sunlight conditions were allowed in the surface of the tanks, to resemble those of real infiltration basins.

Three water ports were installed at the wall of each tank, at depths 4, 18 and 38 cm, to measure piezometric head differences (Fig. S1). Piezometric head measurements were taken from day 19 (earlier values could not be recorded due to technical limitations) with 2-4 day intervals, for a total of 26 sampling dates. From these measurements, hydraulic conductivity (K) values were computed corresponding to depth segments limited by contiguous water ports: surface to 5 cm; 5 to 20 cm; 20 to 40 cm, as well as an overall value (surface to 40 cm depth).

Sand samplings were performed periodically for a total of 9 sampling dates, always around noon (11 – 12 am), and under similar weather conditions. Sand samples were obtained using a sediment core sampler (Eijkelkamp 04.23.SA) and each core was sliced in three depth layers (0-4 cm denoted as “surface sand”, 18-22 cm as “20 cm depth sand”, and 36-40 cm as “40 cm depth sand”). Each layer was homogenized, and subsamples of 1 cm³ were collected using an uncapped syringe, and kept frozen (-20 °C) until analysis to determine algae (chlorophyll-a) and EPS biomass content. For bacterial density and viability determination, samples were kept at 4 °C and transported to the laboratory to proceed with the analysis on the same day. Extracellular enzyme activities (EEAs) in the biofilm were measured in fresh on days 47 and 105. After each sand sampling, one methacrylate empty column was placed at all sampling points to avoid sand collapse, in an attempt to minimize the disruption of the flow field.
2.2. Hydraulic conductivity

Hydraulic conductivity, K [in LT\(^{-1}\)] was calculated using Darcy’s law at the segments already mentioned. For any given two sampling depths, A and B, \( K_{(A-B)} = \frac{Q \cdot \Delta L_{A-B}}{\Delta h \cdot S} \), where \( \Delta h \) is the piezometric head difference between the two points [L], \( \Delta L \) the length of the depth interval [L], S the cross-section area (= 0.4654 [L\(^2\)]), and Q the flow rate [L\(^3\)T\(^{-1}\)]. K values were calculated at different times, and those corresponding to the first sampling time are indicated as \( K_0 \) and reported in Table S1. Values throughout the text are reported normalized by \( K_0 \), therefore different for each system.

2.3. Biofilm biomass: Algal biomass (Chlorophyll-a content)

Chlorophyll-a (chl-a) was measured as a proxy for algal biomass. Chl-a concentrations were determined as described by Jeffrey and Humphrey (1975). Acetone 90 % (10 ml) was added to each sand sample to extract chl-a, and samples were kept in the dark for 8-12 h at 4 °C. Sand samples were sonicated and filtered (GF/C, 1.4 µm, 47 mm). Absorbance was measured at 430, 665 and 750 nm. Results are reported as µg of chl-a · g dry weight (DW\(^{-1}\)). Algal biomass concentrations were also expressed in µg C · g DW\(^{-1}\) by using the conversion factor of Chl:C of 0.021 (Li et al. 2010).

Complementarily, Margalef pigment index (Margalef, 1983) was calculated from the ratio Abs430/Abs665; the higher the value of Margalef index indicates the higher presence of photosynthetic degraded pigments and thus may indicate algal degradation.

2.4. Biofilm biomass: Content of polysaccharides in EPS

EPS were extracted by a cation exchange resin (CER) which is capable of extracting both colloidal and bound EPS fractions. The content of polysaccharides was measured spectrophotometrically following the protocol described by Dubois et al. (1956). Each individual sample was placed in an Eppendorf with 1 ml of Milli-Q water plus 0.3 g of
conditioned CER. After careful shaking, it was incubated with ice for 1 h in a shaker (250 rpm), and then centrifuged (11 000 rpm) for 15 min at 4 °C. The supernatant (500 µl) was pipetted into glass tubes. For each glass tube, a phenol solution (12.5 µl, 80% w/w) was added, shook carefully, followed by an addition of H₂SO₄ (1.25 ml, 95.5%) and capped. After 10 min, samples were carefully shaken and incubated for 10 min in a water bath (30 °C). Absorbance (485 nm) was measured in a spectrophotometer. Results are given in µg glucose-equivalents·g DW⁻¹. EPS content was also expressed as µg C·g DW⁻¹ considering all EPS was built of glucose polysaccharides. Polysaccharides have been proved to be the main component of EPS in sands (Xia et al. 2016; Xia et al. 2014; Hoffmann and Gunkel, 2011).

2.5. Biofilm biomass: Bacterial density and viability

Bacterial density was determined by flow cytometry (FACSCalibur, Becton Dickinson) following a protocol adapted from Amalfitano et al. (2009). Sand samples were placed in glass vials and a detaching solution (10 ml) was added. The detaching solution consists of NaCl (130 mM), Na₂HPO₄ (7mM), NaH₂PO₄ (3mM), formaldehyde (37 %), sodium pyrophosphate decahydrate 99% (0.1 % final concentration) and tween 20 (0.5% final concentration). Samples were then shaken for 30 min (150 rpm) at dark and room temperature conditions, left 10 min at 4 °C, and sonicated with ice during two cycles of 1 min and gently shaken. Samples were left for 5 min for sedimentation of larger particles and supernatant (1 ml) was placed in an Eppendorf. Nycodenz (1 ml) was added to the bottom of the Eppendorf and samples were centrifuged (14 000 rpm) for 90 min at 4 °C. Purified extract (400 µl) was stained with Syto13 (4 µl, Fisher, 5 µM solution) and incubated in the dark for 30 min. Stained samples were counted using flow cytometry. To normalize fluorescence data, a bead solution (10 µl of 10⁶ beads·ml⁻¹
Bacterial viability was determined also by flow cytometry (FACSCalibur, Becton Dickinson). Pyrophosphate (5 ml, 50 mM - Quéric et al. (2004) ) was added to each fresh sand sample and incubated for 15 min at room temperature and softly shaken, and then sonicated for 1 min with ice to avoid cell disruption (Amalfitano and Fazi, 2008).

A subsample of the extract (1 ml) was diluted with 0.2 µm filtered inlet water (1:10).

Diluted extract (400 µl) was stained with propidium iodide and Syto 9 (3 µl, BacLight Bacterial Viability Kit) (Falcioni et al. 2006). Samples were incubated in the dark for 15 min. According to Falcioni et al. (2006), to normalize fluorescent data a bead solution (10 µl of 10^6 beads · ml⁻¹, Fisher 1.0 µm) was added to the samples in a known concentration. Bacteria Live/Dead ratio was then calculated.

Density of live bacteria for each sample was calculated dividing the live/dead ratio by the live/dead ratio plus 1 and then multiplying by the bacterial density. Results are given as number of live cells·10^6·g DW⁻¹. Density of dead bacteria was calculated dividing 1 by the live/dead ratio plus 1 and then multiplying by the density of bacteria. Results are given as number of dead cells·10^6·g DW⁻¹. Data from live and dead bacteria densities were transformed to carbon units (µg C·g DW⁻¹) by using the conversion factor of 1.7 gC (x 10⁻¹³) /cell (Bratbak, 1985).

2.6. Biofilm activity: Extracellular enzyme activities

Extracellular enzyme activities (EEAs) β-glucosidase (EC 3.2.1.21), phosphatase (EC 3.1.3.1) and leucine-aminopeptidase (EC 3.4.11.1) were measured using fluorescent-linked artificial substrates (Methylumbelliferyl (MUF)-β-D-glucopyranoside, MUF-β-D-xyloside, MUF-phosphate, and L-leucine-7-amido-4-methylcoumarin hydrochloride (Leu-AMC, Sigma-Aldrich). All enzyme activities were measured under saturating
conditions (0.3 mM). Sand samples (1 cm³) were placed in falcon tubes with filtered (nylon filters 0.2 µm, Whatmann) synthetic water (4 ml) and artificial substrate (120 µl). A blank for each artificial substrate was prepared with autoclaved Milli-Q water to determine the abiotic hydrolysis of the substrate itself. Samples and blanks were incubated for 1 hour in the dark with agitation; then, glycine buffer (4 ml, pH 10.4) was added to each falcon tube to stop the reaction and maximize MUF and AMC fluorescence. Samples were centrifuged (2000 g) for 2 minutes, and the supernatant (350 µl) of each sample was placed into a 96 wells black plate (Greiner bio-one) for measuring the fluorescence (excitation/emission wavelengths of 365/455 –MUF- and 364/445 –AMC-) in a fluorimeter plate reader (Tecan, infinite M200 Pro). To link fluorescence data and extracellular enzyme activities concentration, MUF and AMC standards were prepared. Results are expressed as nmol MUF·gDW⁻¹·h⁻¹ and nmol AMC·gDW⁻¹·h⁻¹.

2.7. Statistical analysis

Biomass and EEAs differences in depth were analyzed with ANOVA (factor: depth) and further Tukey post-hoc analysis. ANOVA (factor: system) was also applied at each depth to determine significant differences of biofilm parameters depending on GSDs. Dynamics of biomass data measured at each depth and system were fitted with LOcally Estimated Scatterplot Smoothing –LOESS curve fitting- (Loess stats package), (n = 27 for data from the top and mid layers, n = 24 for the bottom layer). Regular sequences of time were generated for further interpolation at each LOESS curve to estimate continuous data over time (frequency = 7 days). With the regular sequences created with the LOESS curve fitting, comparison of biomass dynamics between systems and among depths were performed by using the cross-correlation function estimation (stats package ccf). Temporal K/K₀ values were also LOESS curve fitted to visualize system
dynamics (n = 75) at each depth layer. ANOVA analysis was performed for hydraulic conductivity reduction data (K/K₀) at each depth layer separately (factor: system) to detect significant differences between GSDs. Multivariate analysis with biofilm and K/K₀ data was performed through principal component analysis (PCA) (ggplot in ggplot2 package). Previously, data for the multivariate analysis was standardized separately for each depth (subtracting the mean and dividing by the standard deviation). Carbon contribution of each biofilm-component to the overall C content in sands was plotted in a percentage stacked bar chart (ggplot in ggplot2 package). All statistical analyses and plots have been performed using R software (version 3.1.1).

3. Results

Biofilm structural components (algae, EPS, live and dead bacteria) and biofilm EEAs (β-glucosidase, phosphatase and leucine-aminopeptidase) displayed a vertical decreasing gradient, with the highest values measured in the top layer (values in the mid and bottom layers were at least one order of magnitude smaller than those measured at the top one -Fig. 2, Fig. 3-). In the top layer, no differences in biomass were observed between systems except for higher density of live bacteria in the CF system. In the mid layer, algae and bacterial density (both live and dead) were higher in CF than in F. In the bottom layer, live and dead bacteria densities were higher in CF system than in F. Complementarily, Margalef pigment index showed an increasing trend in depth in both systems (Table S2).
Figure 2 Biofilm structural parameters in depth (top and mid depths based on n = 9 data; bottom n = 8). Capital letters (A, B) indicate statistically significant differences in depth (ANOVA, factor: depth, p < 0.05), asterisks indicate statistically significant higher biomass values in CF than in F (ANOVA, factor: system, p < 0.05).

With regards to EEAs in the top layer, both systems showed similar activity (per units of dry weight and per live bacteria density) (Fig. 3). In the mid layer, CF showed higher β-glucosidase activity (per units of dry weight and per live bacteria density), leucine-aminopeptidase activity (per units of dry weight and per live bacteria density) and phosphatase activity (per units of dry weight) than F. The CF system also showed higher leucine-aminopeptidase activity in the bottom layer (per units of dry weight and per live bacteria density) compared to F.

Extracellular enzyme activities

<table>
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<th>EEAs per dry weight</th>
<th>EEAs per live bacteria</th>
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</thead>
</table>


Figure 3 Extracellular enzyme activities in depth (data from days 47 and 105). Capital letters (A, B) indicate statistically significant differences in depth (ANOVA, factor: depth, p < 0.05). Asterisks indicate statistically significant higher EEA values in CF than in F (ANOVA, factor: system, p < 0.05). β-glu: β-glucosidase; leu: leucine-aminopeptidase; phos: phosphatase.

Biofilm colonization of algae, EPS, live bacteria and dead bacteria in the top layer showed similar dynamics in both systems (all correlations were significant, R² > 0.5, Fig. 4). Algal biomass and EPS concentrations showed an increasing trend until day 40.
when they almost stabilized; contrarily, bacterial density (both live and dead) reached peak values at around day 30, followed by a decrease. However, algal dynamics showed a slight delay in the CF system compared to F (the exact value cannot be given due to the sampling intervals, but as an interval, here 0-7 days). Furthermore, algae and EPS showed an increasing trend around the end of the experiment in CF, indicating that stationarity might not had been achieved.

![Algal biomass](image1)
![EPS](image2)
![Live bacteria](image3)
![Dead bacteria](image4)

**Figure 4** Colonization dynamics of algae, EPS, live bacteria and dead bacteria densities measured in the top layer at each system. Solid lines show LOESS curve fitting (n = 27) for each system. R² values indicate temporal dynamics linear correlation between systems. R² critical = 0.5; values below indicate no significant correlation in biofilm dynamics between systems.

In depth, biofilm accumulation dynamics (see Fig. S2 and Fig. S3 in the supplementary material) arise from the conjunction of biofilm growing in deep layers and biomass detachment from upper layers, transported downwards, and then reattached to the pore network downwards. Biofilm depth-dynamics were studied by statistical cross-
correlations among depths (Table 1). Algae, live bacteria and dead bacteria present at 20 cm depth showed positive correlation with the values measured in the top layer in both systems (Table 1). Bacterial density correlations showed no lag (no delay) but algal biomass values at 20 cm depth displayed a delay compared to those in the top layer, suggesting biomass detachment from the top layer and further downward transport of the autotrophic biomass in both systems. Regarding EPS, no correlations between surface and 20 cm depth were found in the case of the CF system, but in the F system a 0-7 day lag-phase was found, suggesting EPS detachment from the top layer and accumulation in depth in the F system, but not in the CF one.

Focusing on correlations from the different variables measured at depths 20 and 40 cm, the F system showed a strong correlation in depth-dynamics for all the parameters studied (algae, EPS, live bacteria and dead bacteria) (Table 1), indicating similar accumulation dynamics in depth. However, the CF system showed positive correlation on live and dead bacteria dynamics measured at 20 and 40 cm depth –with a lag phase around 7 days at 40 cm depth -, but no depth-correlation was reported for algae or EPS.
Table 1 Cross-correlations of biofilm parameters among depths\textsuperscript{1}.

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top $\rightarrow$ Mid</td>
<td>$R^2 = 0.50$ Lag: 7 days</td>
<td>$R^2 = 0.73$ Lag: 7 days</td>
</tr>
<tr>
<td>n = 14 critical $R^2 = 0.5$</td>
<td>$R^2 &lt; 0.5$ Lag: -</td>
<td>$R^2 = 0.61$ Lag: 0-7 days</td>
</tr>
<tr>
<td>Algae</td>
<td>$R^2 = 0.87$ Lag: 0 days</td>
<td>$R^2 = 0.92$ Lag: 0 days</td>
</tr>
<tr>
<td>Live Bacteria</td>
<td>$R^2 = 0.85$ Lag: 0 days</td>
<td>$R^2 = 0.83$ Lag: 0 days</td>
</tr>
<tr>
<td>Dead Bacteria</td>
<td>$R^2 &lt; 0.55$ Lag: -</td>
<td>$R^2 = 0.72$ Lag: 0 days</td>
</tr>
<tr>
<td>Mid $\rightarrow$ Bottom</td>
<td>$R^2 &lt; 0.55$ Lag: -</td>
<td>$R^2 = 0.63$ Lag: 0 days</td>
</tr>
<tr>
<td>n = 12 critical $R^2 = 0.55$</td>
<td>$R^2 = 0.75$ Lag: 7 days</td>
<td>$R^2 = 0.95$ Lag: 0 days</td>
</tr>
<tr>
<td>Algae</td>
<td>$R^2 = 0.77$ Lag: 7 days</td>
<td>$R^2 = 0.94$ Lag: 0 days</td>
</tr>
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\textsuperscript{1}Values in bold indicate significant correlation among depths at each system for each parameter.

Lag phase corresponds to the day at which the maximum correlation is achieved.

Hydraulic conductivity showed high variability over time resulting in $K/K_0$ oscillations (Fig. 5). The highest effect of bioclogging upon hydraulic conductivity reduction was found in the top 5 cm of infiltration (Fig. 5a), where $K/K_0$ measurements ranged from 0.43 – 0.76 (CF) and from 0.32 – 0.69 (F), indicating a maximum hydraulic conductivity decrease of 57% in CF and 68% in F. From 5 to 20 cm depth (Fig. 5b), $K/K_0$ ranged from 0.46-0.96 in CF and from 0.77-1.17 in F (maximum K reduction of 54% in CF and 23% in F). Last, from 20 to 40 cm depth (Fig. 5c) $K/K_0$ values in CF ranged from 0.89 – 1.04 and in F from 0.69 – 0.93 (maximum K reductions of 11% and 31% in CF and F, respectively). When the system was analyzed as a whole (Fig. 5d), the F system showed a maximum K reduction of 47% and CF of 29%. ANOVA results showed that the F system developed higher bioclogging than CF in the upper and bottom layers and overall, while the opposite happened in the middle layer.
Figure 5 Left: Hydraulic conductivity variations (K/K₀) over time measured at different depths (a: 0 - 5 cm, b: 5 - 20 cm, c: 20 - 40 cm and d: 0 - 40 cm) fitted with LOESS curve fitting (n = 75 for each curve) and polygon bounds indicating 95% of data lying assuming a normal distribution for each curve. Dark lines: CF; light lines: F. Right: boxplots of measured data (the dashed lines indicate K=K₀). Asterisks indicate the system with higher bioclogging at each layer (ANOVA, factor: system, p < 0.05).

Figure 6 displays the results of the PCA analyses for each depth including hydraulic conductivity variations and biofilm representative parameters. In the top layer (upper 5 cm), both CF and F systems showed similar responses of biological and hydraulic...
parameters (Fig. 6a): variations in K closely related to algal biomass and EPS content while densities of live and dead bacteria were uncorrelated to K variations or to EPS and algae concentrations. Clear differences were found between systems at 20 cm depth (Fig. 6b). From 5 to 20 cm depth, lower values of K/K₀ in the CF system correlated to higher bacterial and algal biomass at 20 cm depth compared to the F system. In the bottom layer, lower K/K₀ values in F were strongly correlated to EPS concentration in depth (Fig. 6c).

![PCA analysis](image)

**Figure 6** Plot of the PCA analysis including hydraulic and biofilm structural parameters at different depths (top layer: 0 - 5 cm, mid: 5 - 20 cm, bottom: 20 - 40 cm). LB: Live bacteria; DB: Dead bacteria; chl: chlorophyll-a as a proxy of algal biomass.
Contribution of each biofilm structural component to the overall biofilm organic carbon showed that algae contributed the most to the overall organic carbon measured in sands, decreasing this contribution from the top (\(\approx 90\%\) of total carbon) to the mid and bottom layers where it still represented 50\% of the total organic carbon content. The contribution of EPS and dead and live bacteria increased from top to mid and bottom layers in both systems. In depth, EPS contributed \(\approx 30\%\), dead bacteria \(\approx 25\%\) and live bacteria \(\approx 10\%\) of the overall biofilm organic carbon, at most (Fig. S4).

4. Discussion

4.1 Biofilm colonization in different grain-sized porous media

Decreasing trends of biomass and biofilm activity in depth have been widely reported in porous media (Yan et al. 2017; Freixa et al. 2016; Mermillod-Blondin et al. 2005), mainly attributed to a decrease in the availability of nutrients in depth, thus constraining biomass growth (Battin and Sengschmitt, 1999). Disregarding temporal fluctuations explained by biofilm attachment, adhesion, proliferation, maturation and detachment phases (Lappin-Scott and Costerton, 1989)-, both fine (0.075 - 0.25 mm) and coarse (0.9 – 1.2 mm) sands showed similar ability for biofilm colonization since both systems achieved similar overall biomass densities in the top layer, excepting greater live bacteria accumulating in CF. Although we did not measure bacterial density in the inlet water, other studies showed that WWTPs outflow water carry bacterial densities of around \(10^8\) cells·ml\(^{-1}\) (e.g., Vivas et al. 2017); this implies that the larger hydraulic conductivity (and consequently higher bacterial loads) in the bilayer CF system with respect to the monolayer F one would result in the largest bacterial density in the former system.

However, biofilm colonization dynamics in the top layer were distinct in both systems. Biofilm colonization occurred earlier in the monolayer F system than in the CF one,
possibly driven by the combination of low fluxes and high surface area in the former
(Donlan, 2002) which could have favored biomass attachment and accumulation (Fang et al. 2017).

Biofilm growth asymptotically approaches steady state as the result of the balance
between biomass decay/detachment and growth (Bottero et al. 2013). Linked to this, the
increasing trend in algae, EPS and live bacteria densities at the end of the experiment in
the bilayer CF system suggests a transient effect due to still existing available space for
biomass growth mostly in the coarse sand. On the contrary, biofilm in the top layer of
the monolayer F system could have reached a maturity state resulting in equilibrium
between the biomass detachment and accrual terms.

4.2 Grain-size distribution of the porous media modulating deep bioclogging: biofilm
attachment, detachment and vertical transport

Differences observed on biomass dynamics in depth between systems could be due to
biomass depth-accumulation being a conjunction between solute availability in depth,
flux rates, and detachment/attachment dynamics, all influenced by GSDs. Linked to
this, EPS accumulation in depth is driven both by biomass downward transport and by
EPS bacterial production. Interestingly, EPS detachment from the top layer and further
transport seems to be the main EPS accumulation pathway in depth in the monolayer F
system. Contrarily, no EPS correlation in depth in the bilayer CF system as well as
higher density of live bacteria suggested EPS production by bacteria to be the main
pathway for EPS accumulation in depth in the bilayer system.

High flow velocities result in thin but strong biofilm matrices, while under low fluxes,
thicker and less cohesive (more detachable) biofilms are observed (Gerbersdorf and
Wieprecht 2015, Laspidou and Rittmann 2002, Graba et al. 2013). Thus, under high
flow, and correspondingly high shear forces (as expected in the bilayer CF system), a
denser, cohesive, and more stable biofilm is likely to be produced because of strong adherence to the EPS matrix in comparison to biofilms subjected to low shear forces (Oyebamiji et al. 2018), such as the monolayer F system. Furthermore, biofilm reaching steady state in the top layer (F system) could have enhanced biofilm detachment and transport in depth, following Liu and Tay (2002), who observed that mature biofilms were strongly affected by hydrodynamic shear stresses. This reinforces the idea that combined hydraulic and biofilm dynamics, both modulated by GSD, may strongly influence biofilm detachment in porous media.

It is widely accepted that advection is the main mechanism for transport of dissolved and particulate matter (as well as detached biofilm) through the interstitial space (e.g., Zhong et al. 2017; Huettel et al. 1998), so that the effect of diffusion can be neglected. Nonetheless, heterogeneity of the pore structure results in preferential flows during infiltration (e.g., Rubol et al., 2014) being the source of hydrodynamic dispersion and driving mixing (Rodriguez-Escals et al., 2017). Heterogeneity is enhanced at interfaces between materials (e.g., coarse and fine sands), where a large distribution of local velocities spanning several orders of magnitude can be found. Consequently dispersion, forced by the presence of a strong variation in local velocities, could have produced the conditions to depth-trapping algal and bacterial biomass hindering advective biomass transport from the interface downwards in the bilayer CF system.

All these biofilm dynamics showed great implications on the occurrence of bioclogging. Specifically, the monolayer F system showed higher degree of bioclogging in the top layer and in the bottom one. This could be due to the monolayer F system developing similar density of biomass in the top layer (specifically EPS and algae which are the main contributors of surface bioclogging) but lower pore sizes (also slightly lower porosity) than the bilayer CF system, thus promoting surface bioclogging. Yet, the
expected biofilm is non-cohesive, and large amounts are detached and transported downward, allowing for additional pore space to grow additional biofilm on top while also detached biomass having the potential to accumulate in depth, causing deep bioclogging. According to Watnick and Kolter (2000), detached biomass cause the spreading of biofilms along the advective path, as they are the primary mechanism for translocation from one surface to another.

Our results indicate that biofilm biomass can reach depths of at least 40 cm in both fine (0.075 – 0.25 mm) and coarse (0.9 – 1.2 mm) sands. Specifically, EPS concentrations at 40 cm depth (accounting only for 15-20 % of the overall carbon biomass in the biofilm) seem to be the main cause of deep bioclogging in the monolayer F system. Even though EPS densities in the bottom layer were similar in both systems, EPS quality could be different between systems thus showing a different effect on bioclogging. Note that although algal biomass at 40 cm depth represented the main structural component of biofilms in terms of C, it does not show direct implications on deep clogging possibly due to its degraded state in the bottom layers. In depth, the presence of degraded algae is expected due to the lack of incident light, thus limiting its growth, and validated by the increasing values of the Margalef pigment index with depth indicating an increase in chlorophyll degradation products.

4.3 A bilayer coarse-fine distribution: minimizing bioclogging in infiltration systems

Maybe less intuitive is the potential of the interface coarse-fine to minimize the occurrence of deep clogging in infiltration systems. Even though the interface becomes the location for biomass accumulation, our results suggest that it also results in a hot-spot of microbial activity (as highlighted by Perujo et al. 2017) which causes the presence of high EEA values in depth and high live bacteria densities, thus helping maintaining the hydraulic conductivity values of the porous media with time.
On the contrary, the monolayer fine system exhibited higher bioclogging in all layers; so, as a whole, it exhibited large overall (non-localized) bioclogging due possibly to the combination of strong biomass detaching and recolonization in the top layer. Furthermore, in the monolayer system, low EEA values in depth, low bacterial viability and low availability of solutes could contribute to high biomass and organic matter accumulation in depth in the long term.

4.4 Relevance of the Findings and Limitations of the Study
Results from the present study state that bioclogging occurrence in infiltration systems can be reduced by controlling the characteristics of filter media such as GSD as well as by understanding biofilm dynamics in surface and in depth. Altogether, this study may help improving the design and maximizing the performance of infiltration systems used as tertiary water treatment systems.

Successful performance of infiltration systems requires maintaining relatively high hydraulic conductivity values of the system as a whole to convey water efficiently from the surface downwards during periods of system operation (Racz et al. 2012); thus it is important to reduce the land use necessary for their application (Bouwer, 2002), the maintenance operations, and the overall cost. By adding a layer of coarse sand on top in systems where the natural material is fine sands: (1) we are able to infiltrate higher volume of water per surface area unit than in a monolayer system (only fine material); (2) surface and deep bioclogging are minimized, increasing the longevity of the infiltration system and keeping the high infiltrating volumes for longer times, and (3) water treatment efficiency is maintained, as previously reported in Perujo et al. (2018).

This study could also benefit further understanding and provide useful modelling tools to capture the effects of the biofilm growing stage on biofilm dynamics such as attachment/detachment influenced by hydrodynamics and GSD of the porous media and
their influence not only on surface but also on deep bioclogging. Agreeing with Smith et al. (2018), the lack of data and understanding of microbial processes has challenged so far the incorporation of microbial ecology into bioclogging models.

We also observed that biofilm-component dynamics are not the same for bacteria, EPS and algae, so differentiation between these parameters should be done in future studies addressing bioclogging. Also, in the future it would be interesting to study the role of EPS fractions on deep bioclogging linked to EEAs. Accordingly, EPS can be classified in two main fractions: colloidal EPS that is soluble in water and secreted in the vicinity of cells, and bound EPSs that is tightly attached to the cell walls (Chen et al. 2017). Different EPS fractions may have distinct impacts on driving hydrological changes within the porous media.

**Conclusions**

From this experimental study we could state that biofilm structural parameters showed different dynamics and implications on bioclogging in infiltration systems. In particular, grain-size distribution along the vertical has a strong influence in biomass attachment. In fine sands, higher availability of surface area as well as lower fluxes could favor the initial biofilm formation phase (attachment) as compared with coarse sands. This translates to an early biofilm formation in the monolayer fine system considered in the field experiment analyzed in this work, which resulted in an early surface bioclogging (also favored by the smaller pore sizes, easily colonizable). At the same time, this early biofilm formation resulted in an early maturation stage of the biofilm, which promoted biomass detachment from upper layers and further recolonization of the topsoil by new biomass. Further, the presence of a homogeneous grain size enhanced the transport of biomass in depth modulating not only the occurrence of surface bioclogging but also deep bioclogging.
Contrarily, in the bilayer coarse-fine system we observed an active biofilm with high activity per surface unit (which includes organic matter degradation and thus helps to control bioclogging). The interface in the bilayer system acted as a biomass filter with a hot-spot of microbial activity which minimized deep bioclogging. This is due to this interface trapped biomass in a middle-depth where availability of solutes is higher promoting microbial activity in the interface with additional implications in deeper layers. As a whole, a bilayer system with an upper layer of coarse sands (around 1 mm) showed similar biofilm colonization than fine sands (0.075 – 0.250 mm) and higher overall biofilm activity minimizing bioclogging, and therefore, having the potential to increase the longevity and efficiency of infiltration systems.

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Supporting Information. Scheme and photograph of the experimental design used for piezometric head measurements; accumulation dynamics of biofilm-structural components in the mid and bottom layers; percentage of carbon content of each of the biofilm-structural components from the total biofilm carbon; values of hydraulic conductivity measured at day 19 at each depth layer; Margalef pigment index calculated for each system and depth.
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