Dynamics of Extracellular Polymeric Substances (EPS) Derived from Biofilm in On-Site Wastewater Filtration Reactors

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Abstract
Extracellular polymeric substances (EPS) from biofilters (packed with river sand (RS) and crushed aggregate (CA)) set in on-site wastewater treatment systems are characterized over 360 days within bed thickness to investigate its correlation with the enrichment process. Biochemical component contents are monitored. Moreover, humic and protein-like compounds are characterized by means of Size Exclusion Chromatography (SEC) coupled with fluorescence. During the biomass enrichment phase, EPS biochemical components increase at the top of the biofilter (protein enrichment factor >70%). The protein-like components exhibit a very high MW fraction (apparent molecular weight (aMW) >1,000kDa), which may contribute to cell aggregation. Humic-like substances show similar SEC fingerprints to those of the feed water (aMW<6kDa) and are perhaps being metabolized at around Day 210 (as evidenced by a lower aMW). Only the dynamic polysaccharide partition in EPS differs between biofilters, with an increase for CA and a decrease for RS. Within the filtration bed thickness, lower biomass with a higher EPS content is observed, and the polysaccharide fraction increases by a factor of 2. Protein-like components exhibit a very high MW fraction at a lower magnitude. The clogging risk due to the presence of polysaccharides or their combination with high aMW extracellular proteins should be considered for alternative materials for onsite wastewater filtration system.

INTRODUCTION
The extracellular matrix of biofilm contains a "gel-like" structure. It is composed of major biochemical components which are either tightly or loosely bound to the cells, such as proteins, polysaccharides, humic-like substances [1,2], metabolic wastes, and absorbed substrates and minerals [3-5]. It is conceivable that intracellular material excretion is a survival mechanism under unfavorable conditions, such as enhanced extracellular enzymatic activity and/or other mechanisms which facilitate cell aggregation or surface attachment [6-8].

Protein and polysaccharide EPS contents had been tracked as potential indicators of the state of the biofilm: protein content increases with the formation and stability of aerobic granules [9], whereas Ahimou et al. [10], showed that the level of biofilm cohesive energy is strongly correlated with polysaccharide content. The humic-like substances (HS-like) are believed to be exogenous compounds captured in the biofilm before undergoing repolymerization [11]. However, Guo et al. [12], found that humic acid-like substances detected through three-dimensional spectrofluorimetry appear during aerobic granulation. Some authors have pointed out that these HS-like can form through another pathway: following the degradation of macromolecules under microbial attack (such as carbohydrates and proteins), the refractory compounds or biopolymers are selectively transformed to produce the high MW precursor of humin. The molecules then become smaller during the additional oxidation process [13].

In this study we want especially to follow the humic like substance and protein evolution of EPS component from biofilm involved in wastewater biotreatment and whose increase stops the bioprocess. In this we chose biofilm of biofilter from "on-site wastewater treatment systems" (OWTS) [14]. The structural characteristics of biofilm (i.e. the EPS matrix) take into account the environment of a porous medium and tend to further reduce the limited space between pores, until biofilm development reaches stabilization, which may "clog" porous media provided by river sand or other materials, e.g. polysaccharides [15,16].

Previous research on biofilms extracted from filtration reactors has mainly dealt with the biological dogging tied to biomass expansion and with the microbial community on the filter medium, i.e. total organic accumulation [15,17], including the main biofilm components. Proteins and polysaccharides exhibit a linear correlation with operating time and a decreasing abundance with depth [18]. It appears that among these organic compounds, the loose "slime-like" exopolymers may cause the observed drop in hydraulic conductivity, while the cells exert...
MATERIALS AND METHODS

Batch experiments (cylindrical filtration reactors)

Two filtration reactors, 70 cm high and 30 cm in diameter with sampling ports, were packed with river sand and crushed aggregate, respectively (Figure 1). Septic effluent stored in a mixed tank with replenishment every 7 days was then fed to the reactors. These reactors were fed discontinuously, at a hydraulic loading rate of 12 cm/day in 10 daily batches. The feed-rest condition induces liquid passage, which may involve varying the O$_2$ level. An optical oxygen sensor was used to estimate the O$_2$ gas variations in each reactor (OXROB10, Fire Sting O$_2$, with Pyroscience sensor technology). A saturated/unsaturated gas phase alternation was found with the river sand reactor. However, the variation in oxygen gas was lower for the reactor with crushed aggregate.

Packing material characteristics

The filter materials were analyzed before column packing, given that the river sand and crushed aggregate differed in composition and that they involved distinct treatment processes at the quarries. The two materials were studied and compared in terms of their respective particle distribution, mineralogical and physical characteristics. Their hydraulic and hydrodynamic properties were assessed using two post-packing filtration reactors [21] (Table 1).

Feed water: Septic effluent characteristics

The feed water was collected from the septic effluent settling tank. All its major features were monitored throughout the operating period. The average and extreme values of each characteristic are reported in Table 2.

Total biomass and EPS extraction

Three samplings and extractions were carried out on Days 60, 210 and 360 for the top layer of the medium. At the end of the operating period (i.e. 360 days), extractions were also performed on the various layers in the two filtration reactors (5 cm, 10-15 cm and 30 cm). The total biomass was extracted as quickly as possible (<24h) both by heating to 80°C during 30 minutes for the colorimetric analysis (proteins, HS-like, polysaccharides and nucleic acids) and by sonication at 4°C during 60 minutes for the Size Exclusion Chromatography (SEC) fingerprint analysis. The EPS was extracted by sonication at 4°C over 5 minutes for both the colorimetric and SEC fingerprint analyses. The nucleic acids were assessed with a series of increasingly long ultrasound extractions: after 5 minutes, the nucleic acid contents exhibited a significant increase within the filter media samples.

Total biomass, feed water and EPS characterization

The biochemical components of biomass, EPS and feed water were quantified through the colorimetric methods: proteins and humic-like (HS-like) substances using the modified Lowry method in introducing Folin’s reagent [1]; polysaccharides using Dubois’ method; and nucleic acids using Burton’s method [23,24]. Nucleic acids are present to control EPS extraction (Liu et al., 2004). The EPS extracted during this study exhibited a relatively weak nucleic acid content, which suggests that this EPS was not contaminated by significant amounts of intracellular materials [25].

The apparent molecular weight (aMW) distributions of biomass proteins and humic substances were analyzed with High Pressure Size Exclusion Chromatography (HPSEC) (Merck Hitachi LA Chrom Chromatograph), coupled with fluorescence detection. The high molecular weight separation was performed with the Agilent column (BioSec, 300A, 5-1,250 kDa), while the low MW separation was executed with the BioSec 100A Agilent column (0.1-100kDa). All columns were placed in series for the separation improvement step [26]. The mobile phase was composed of a 150 mM NaCl and 50 mM phosphate buffer at pH 7.0. The MW were calibrated with six proteins or amino acids, with MW values of: 440000, 155000, 69323, 5777, 362 and 181Da (matching respectively ferritine (Sigma), immunoglobulin G from human serum (Sigma), albumin from bovine serum (Sigma), insulin from porcine pancreas (Fluka), thyroglobulin, thyrotropin-releasing hormone (Fluka), and tyrosine (Fluka)). The logarithm of molecular mass (Log (MW)) was plotted as a function of the elution volume (mL) for the mass calibration curves, i.e.:

\[
\text{Log} \ (MW) = -0.3164 \ Ve + 9.4676 \quad (R^2 = 0.982)
\]

Where, MW is the molecular weight (Da), and Ve is the elution volume (mL). The permeation volume determined with Na$_2$SO$_4$ equaled 22 mL. The Excitation/Emission (Ex/Em) wavelength fluorescence detection for protein-like (protein tryptophan-like) substances was found to be 222/330 nm, while the value
Table 1: Characteristics of river sand (RS) and crushed aggregate (CA) when used as the main packing material.

<table>
<thead>
<tr>
<th>Material</th>
<th>River Sand (RS)</th>
<th>Crushed aggregate (CA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size distribution characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effective size $D_{10}$ (mm)</td>
<td>0.38</td>
<td>0.17</td>
</tr>
<tr>
<td>Average diameter $D_{m}$ (mm)</td>
<td>0.82</td>
<td>1.36</td>
</tr>
<tr>
<td>Uniformity coefficient ($D_{60}/D_{10}$)</td>
<td>2.8</td>
<td>10</td>
</tr>
<tr>
<td>Fine particles % (&lt;0.08 mm %)</td>
<td>0.40%</td>
<td>5.00%</td>
</tr>
<tr>
<td>Physical and chemical characteristics (average values; n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real density (kg/m$^3$)</td>
<td>2525 (± 42)</td>
<td>2438 (± 111)</td>
</tr>
<tr>
<td>Porosity [% [min; max]]</td>
<td>[30%; 33%] (± 1.5%)</td>
<td>[38%; 41%] (± 1.7%)</td>
</tr>
<tr>
<td>Specific surface area (m$^2$/kg)</td>
<td>4.04</td>
<td>2.78</td>
</tr>
<tr>
<td>Hydraulic characteristics (average values; n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated hydraulic conductivity (m/s) [min, max]</td>
<td>[8.25~9.53] × 10$^{-4}$</td>
<td>[2.79~2.88] × 10$^{-4}$</td>
</tr>
<tr>
<td>Water retention capacity after drainage (static water %)</td>
<td>8.40%</td>
<td>12.70%</td>
</tr>
<tr>
<td>Hydraulic Residence Time (hours) (n=1)</td>
<td>35</td>
<td>93</td>
</tr>
<tr>
<td>Variation of $O_2$ gas level at 10 cm [min, max%]</td>
<td>[11, 20]%</td>
<td>[19.2, 19.8]%</td>
</tr>
<tr>
<td>Mineralogical composition (mg/kg of material)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>&lt;5</td>
<td>795</td>
</tr>
<tr>
<td>Mg</td>
<td>74</td>
<td>1535</td>
</tr>
<tr>
<td>Na</td>
<td>&lt;5</td>
<td>295</td>
</tr>
<tr>
<td>K</td>
<td>1370</td>
<td>2356</td>
</tr>
</tbody>
</table>

Table 2: Feed water characteristics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average value</th>
<th>[min; max]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pH$ (n=5)</td>
<td>7.1 (± 0.3)</td>
<td>[6.6; 7.5]</td>
</tr>
<tr>
<td>TSS (mg/L, n=18)</td>
<td>39 (± 11)</td>
<td>[20; 66]</td>
</tr>
<tr>
<td>VSS (mg/L, n=1)</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>COD (mgO/L, n=18)</td>
<td>372 (± 100)</td>
<td>[231; 572]</td>
</tr>
</tbody>
</table>

The chromatograms were established from both fluorescent intensity (volts) and elution volume (mL). Several fractions were identified as the elution volume interval (e.g. 12~18 mL). The fractional area percentage calculation was based on the ratio of the chromatogram area of one fraction to the total chromatogram area. This calculation was performed using the Origin 6.0 software package. The fractional (F) area percentage is defined as follows:

$$F\% = 100 \times \frac{\text{Area of fraction}}{\text{Total area of chromatogram}}$$

RESULTS AND DISCUSSION

Evolution of EPS biochemical characteristics vs. operating time

Biochemical composition: The evolution of EPS percentages in the biomass extracted from the top layer of each filtration reactor on Days 60, 210 and 360 is presented in Table 3 (Part 1). During the implementation process, total biomass increased in the two materials, by factors of 3 and 2 for RS and CA respectively. It is noticed that stationary state of the biomass growth is not achieved yet for two reactors and especially for CA (slowdown state for RS) (Figure 2). This biomass is mostly correlated with protein content: $r^2$ are 0.97 and 0.94 for RS and CA, respectively (and only 0.96 and 0.75 for humic substance; 0.81 and 0.83 for polysaccharides). According to Di Iaconi et al. [28], protein serves as the biomass activity indicator. Nevertheless Regusa et al. [18], shown that polysaccharides also exhibit a linear correlation ($r^2=0.99$) under operating time of only 120 days in subsurface wetland reactors fed with fill and draw cyclic sequence. EPS % increases with time to reach around 30 % of the total biomass at day 360 for the two biofilters. There is no correlation with biomass increase (lower EPS% at Day 210 than at Day 60) during the 360 days (Table 3, Part 1). Whether EPS production reflects higher or lower microbial activity is still open to debate, although it is usually reported that during the exponential growth phase, EPS content increases with time and yet decreases once the stationary phase has been reached [29]. Previous studies also pointed out that the presence of extracellular macromolecules engaged in the process of cell adhesions onto the mineral surface by involving much more complex adhesion behavior [7]. Both EPS percentages exhibit a slight decrease between Days 60 and 210 (Table 3, Part 1). This decrease is simply due to the drop in HS-like substances (Figure 2) from 7.1 to 5.9 mg/kg material in RS and from 15.1 to 7.1 mg/kg material in CA. Humic substances, which are generated from effluent, do not get synthesized. The hypothesis may thus be advanced that biofilm selectively adsorbed humic-like compounds from the feed water which...
is mostly composed with humic substance (21, 62 and 17 % of protein, humic substance and polysaccharides) at a different developmental state, and/or that the HS-like degradation (catabolism) took place in the biofilm not yet at stationary state [13,30]. Nonetheless, the biomass EPS percentage remains somewhat higher for CA, especially at the beginning of the implementation process (Table 3, Part 1). At this same time, a higher biomass increase for RS than for CA is unveiled. The biomass content evolution could be due to a specific surface area higher for RS than for CA (Table 1) or to a higher speed of development. As observed in the material characteristics (Table 1), the CA reactor displays an extended hydraulic residence time and water retention capacity, both of which may due to its fine particle content. The static liquid phase may lead to microenvironments with a weak renewal of nutrients and/or accumulated residues. The hydraulic and hydrodynamic variations could result in low biomass activity (i.e. total protein content in CA) but in a high proportion of EPS in the reduced biomass (as is the case with CA). This low substrate content could favor EPS synthesis [31,32]. Starvation would be the preferred hypothesis over hydrodynamic influence for the increase in EPS content from aerobic granules growing on a zeolite material biofilter [28]. Moreover, changes in environmental conditions could induce a shift in microbial community and/or activity and, subsequently, more EPS-producing content [6,31]. Throughout the entirety of the process, total -N removal (%) was greater for RS than for CA (by a factor of 1.5), which means that denitrifying bacteria are less active or less numerous in the CA biofilter. Previous study concluded that the bacterial retention in saturated porous media is the combined results of various factors, such as solution ionic strength, flow velocity, grain size and surface roughness [33].

The evolution in biochemical component quantities of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA) suggestions that the evolution in extracellular biochemical components undergoes the same process for both materials. Enrichments were pointed in: PN (proteins)-like (with growth factors of 19 and 3 for RS and CA, respectively), HS-like (by factors of 5 and 2 for RS and CA, respectively), and PS (factors of 3 and 5, respectively). The majority of organic matter in an extracellular matrix is composed of proteins and humic compounds which are found in greater proportions in active sludge [1]. Zhang et al. [9], observed that the stability of aerobic granules during biofilm granulation is mediated by PN, while for Ahimou et al. [10], cohesiveness is correlated with PS.

When comparing the evolution of proportions in biochemical components, this same trend is identified with increasing PN percentages (RS: 10~33%; CA: 24~31%) and decreasing HS-

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**Table 3:** Biomass evolution and EPS percentages in the biomass extracted from the top layer of each filtration reactor on Days 60, 210 and 360 (Part 1) and extracted from bed thicknesses on Days 360 (Part 2). c

<table>
<thead>
<tr>
<th>Material</th>
<th>River sand (RS) - top layer</th>
<th>Crushed aggregate (CA) - top layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>60 days</td>
<td>210 days</td>
</tr>
<tr>
<td>Biomass (mg/kg)</td>
<td>96</td>
<td>238</td>
</tr>
<tr>
<td>EPS%</td>
<td>16</td>
<td>9</td>
</tr>
</tbody>
</table>

**Part 2:** Evolution of biomass and EPS% from biomass extracted over the depth of each reactor at Day 360

<table>
<thead>
<tr>
<th>Material</th>
<th>River sand (RS) on Day 360</th>
<th>Crushed aggregate (CA) on Day 360</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (cm)</td>
<td>0-2 cm</td>
<td>5 cm</td>
</tr>
<tr>
<td>Biomass (mg/kg)</td>
<td>303</td>
<td>169</td>
</tr>
<tr>
<td>EPS%</td>
<td>30</td>
<td>25</td>
</tr>
</tbody>
</table>

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**Figure 2** Evolution in biochemical component quantities of biomass and extracted EPS vs. time in both river sand (RS) and crushed aggregate (CA).
like (RS: 47~43% CA: 50~33%). Zhang et al. [9], described such 
enrichment in PN of EPS content during the formation of aerobic 
granules and suggested that an increase in PN might enhance 
neighboring microbial cells and form a cross-linked network 
by attracting organic and inorganic material [6]. However, the 
percentage of PS (polysaccharides) becomes inverted, with 
an increase (18~33%) and decrease (30~18%) for CA and 
RS, respectively. Hence it follows that, EPS in biofilm does 
not necessarily develop similarly on two different filter materials, 
this is especially detectable after long time of process (360 days). 
The difference in EPS biochemical composition may be explained 
by the differing conditions generated in the materials, i.e. porosity 
(both external and internal), fine particle contents and reactor 
hydrodynamics. Moreover, the mineralogical composition (Table 
1) differs substantially with crushed aggregate: higher calcium 
content leads to a greater presence of divalent cations at the 
material surface, with the possibility that divalent cations bind 
with extracellular PS-alginate-like via an ionic link, thus resulting 
in a complex "egg-box" configuration [34,35]. Stabilized PS are 
less influenced by effluent and/or less metabolized. 

Studies showed that proteins possessed high molecular 
weights (45-670 kDa) and may be associated with other 
macromolecules, such as polysaccharides [26,36], which has 
been reported causing the clogging in pilot study of sand filter 
during 95 days of operating [37], and in real scale study of a 
septic system during 10 months [20]. As mentioned above, the 
polysaccharides proportion in EPS was enhanced in CA biofilter. 
Thus a hypothesis may be dawn that with the presence of high 
aMW proteins-like macromolecules and polysaccharides, the 
porous media constructed by such filter material might be 
exposed to the clogging risk for long term operating periods. 

In light of the abovediscussion, similar trends can be observed 
for PN- and HS-like, as recorded in both materials. A follow-up 
qualitative comparison of PN-like and HS-like substances was 
conducted by examining the MW distribution. 

**Protein and HS-like HPSEC fingerprints:** In examining the 
major biofilm components, a further qualitative study on the 
apparent molecular weight (MW) distribution by HPSEC fingerprints of protein-like substances between filter 
materials will be described, and the chromatogram displayed 
in Supplementary data (S1). Moreover, the fractional area 
percentages are presented in Figure 3. 

As observed in the PN-like fingerprints their peak number 
and elution volume differ with incubation time, as well as with 
the one of feed water PN-like compounds. This finding indicates 
that various molecular structures have developed as a result 
of biomass enrichment. Similarly, in Figure 3, the increase 
in the very high MW (VHMW, aMW ≥ 1000kDa) fraction and 
decrease in the very low MW (VLMW, aMW=6 kDa) Fraction of 
PN-like fingerprints were noted in both filter materials after 
60 days of operations. This MW distribution shift may be due 
to the production of new PN-like and their polymerization 
with other organic molecules during the enrichment step [38]. 
Since extracellular proteins contain a considerable amount 
of enzymes, it is assumed that the extracellular enzymatic activity 
might change with the increased biofilm implementation activity. 
This increase in the VHMW fraction may be correlated with the 
aggregation of bacteria in biofilm during biomass enrichment. 

The RS showed a relatively stable evolution in fractional area 
percentages from Day 60 to Day 210. A decrease in the VHMW 
fractional proportion, coupled with an increase in the low MW 
(LMW, 25 kDa<aMW<109 kDa) fractional proportion, could be 
noticed at Day 360. It may be possible to explain the decrease 
in the VHMW fractional proportions by degradation in existing 
compounds at the mature biofilm state [1]. On the other hand, for 
CA, the VHMW fraction increases during the entire 360 days 
of biomass enrichment, thus suggesting a late increase in molecular 
polymerization. Delayed biofilm development may provoke this 
late evolution in the MW shift distribution of PN-like molecules 
in CA. This pattern was also observed in the biochemical 
component evolution for total biomass (Figure 2). It should also 
be noted that between Days 210 and 360, the high MW (HMW: 
109 kDa<aMW<1000 kDa) fractional distributions diminished 
in both materials. This may be due to the degradation of PN-like for 
cell catabolism. 

Thus the presence of, very high or low aMW proteins- 
like macromolecules have been confirmed for both materials. 
However along with the increasing polysaccharides proportion 
in EPS, such as the case of CA, might be an indication of further 
clogging development risk for long term operating biofilters. 

The HS-like fingerprints of the EPS extracted from the two 
materials were also described from Day 60 to Day 360 (Figure 4). 

As mentioned in the previous section, it was confirmed that 
HS-like possessed weak MW (<6 kDa) on Day 360 in both 
materials. The majority fraction (i.e. major peak) varied similarly 
between the two filter materials over the operating period. The 
small peaks located between 20 and 22 mL on Day 60 had moved 
to between 20 and 21 mL by Day 210 and were positioned at 20 mL 
on Day 360. It must be noted, however, that for CA in particular, 
the area under peak evolution exhibited the same trend as the HS- 
like content observed in Figure 4 over time. During this process, 
the aMW of HS-like increased. As previously stated, it is possible 
that the HS-like first absorbed in the biofilm might thus be the 
humic compounds of very weak MW or the HS-like modified 
by the biofilm. Furthermore, the HS-like fingerprints of EPS in 
both materials were similar to those of the feed water. During 
the first process interval, either: i) in considering the hypothesis 
of selective adsorption of HS-like from the effluent, smaller 
molecules are perhaps more easily and quickly adsorbed onto 
the biofilm; or ii) in considering the hypothesis of metabolism of 
HS-like by cells, HS-like from the environment are no longer used 
after implementation (hence they are no longer being degraded). 

**Evolution of EPS biochemical composition with bed 
thickness**

To observe the impact of packing materials on biofilm 
evolution in the deeper sections of the filtration reactors, (i.e. 
where the environment differs from that of the top layer and 
is less rich in substrates), a follow-up study of the vertical EPS 
distribution in filtration beds with different filter materials was 
conducted after 360 days of operations, using the same analytical 
tools. 

**Biochemical composition:** The evolution of biomass and
EPS percentages in the biomass extracted from layers 0-2 cm, 5 cm, 10-15 cm and 30 cm in each biofilter on Day 360 (as calculated from the data available in Figure 5) is shown in Table 3 (Part 2). The biomass in both materials decreased with filter medium depth. Moreover, a higher biomass in RS from the top to the 10-15 cm layer and a lower biomass at 30 cm compared to CA were observed. This same trend had been identified for both types of material packing: increasing EPS percentage in biomass, coupled with a declining biomass over the depth. The EPS percentage remained constant in the same layer and on the same order of magnitude for both materials: about 30% for the top and increasing with depth (up to 38%).

The biomass also decreased with depth due to the presence of fewer cells when a smaller portion of the substrate had penetrated into the depth. As per the previous study (EPS vs. time of evolution process), total biomass components appeared in higher contents from the top to the 10-15 cm layer in RS than in CA. EPS components showed a similar scale, except with a slightly greater number of polysaccharides in CA. Above this level, the increase in EPS percentage compared to its total biomass over the filter medium depth could be due to: i) the downward shift through the depth of the weakly bound soluble EPS; ii) the reduced substrate and/or bacteria community shift (more nitrifying bacteria, which are autotrophic); and iii) a new C/N ratio over the deeper part of the beds. Gao et al. [31], also described how nitrifiers (which appeared due to the new C/N ratio) tend to produce more EPS than heterotrophs within the upper media of an aerobic biofilter. Moreover, as is expected with depth in our study, a low C/N ratio was reported by Durmaz et al. [39], to induce a low EPS content when compared with a ratio of 40. The fine particles in CA should be more noticeable over the
depth than in the top layer, which may also lead to a higher EPS percentage by structurally influencing the biofilm [40].

There were modifications in biochemical component proportions in the deeper part of the filtration beds (Figure 6, 30 cm). CA revealed lower quantities of EPS in proteins than in polysaccharides (in both absolute and relative fractions), with the PN/PS ratio decreasing from 1 in the top layer to 0.1 at 30 cm. For the RS material, PN/PS ratios were typically higher, with a value of 2 in the top layer, around 1 from 5 to 15 cm and 0.5 at 30 cm. In deeper parts of the filtration beds, bacteria feature less substrate. Furthermore, the proportions of polysaccharides in EPS, which increase with medium depth in CA and RS, might be due to the lower accessibility of nutrients (starvation) or to the change in bacterial community, for example, the nitrifying bacteria are considered more in the lower part of filter where the COD is less concentrated, or also to a new C/N ratio in the substrate, or yet to various physical environments [6, 28, 41]. However, Durmaz et al. [39], demonstrated that a lower C/N ratio induces an increase in PN content and a decrease in PS content.

**Vertical evolution of proteins and HS-like fingerprints:**

The MW evolution of PN-like compounds in the 10-15 cm and 30 cm depths was also studied by means of HPSEC fingerprints. Presented in Supplementary data (S2), these results show similar fractions of PN-like EPS to those in the top layer. Moreover, the four major fractions were identified as in Figure 3. The corresponding fractional percentage calculations are shown in Figure 7.

For both materials, the 10-15 cm and 30 cm layers exhibited a lower percentage of VHMW and HMW fractions, and yet it also showed a higher percentage of LMW and VLMW fractions, which indicates that fewer high MW PN-like polymers were formed in the lower part of the filters. These low aMW compounds (<6 kDa) may be amino acid-like, small peptide-like or molecules of similar configurations conveyed by the feed water, and then transported and absorbed onto the deep medium. The LMW and VLMW fractions may also be associated with low MW PN-like molecules, resulting from substrate degradation through the metabolism of microorganisms [42].

The HS-like fingerprint evolution over the depth of two materials was also compared and summarized in Figure 8. These fingerprint chromatograms reveal shapes similar to the two filter materials and feed water which suggests that, HS-like compounds stem from the retention of exogenic organic matter. The HS-like fingerprints also display overlapping peaks with the 10-15 cm and 30 cm samples of both materials. Moreover, they indicate that HS-like may be captured from the feed water and distributed relatively homogeneously inside the filter medium, despite the fact that the types of filter materials and these exogenic HS-like compounds possess similar and low MW (<6 kDa).

**CONCLUSION**

This paper has sought to provide in-depth information regarding the EPS extracted from a biofilter at various enrichment stages during a 360-day period and for different bed thicknesses. Two types of packing materials were used (river sand and crushed aggregate) and a series of quantitative and qualitative analysis were conducted to investigate the differences in EPS characteristics especially for proteins-like and humic substances-like components. The following conclusions could be drawn:

- Overall, the percentages of EPS in biomass increase with biomass enrichment, despite a decrease due to humic-like substances from Day 210. Even though the stationary state of purification performance was reached for both biofilters, the biomass evolved with changing repartitions of proteins, humic substances and polysaccharides in EPS till the end of the operating. During biomass enrichment, the humic-like substance/protein ratio decreases. Meanwhile, within the bed thickness, the polysaccharide/protein ratio rises by Day 360. Moreover, a correlation between the higher percentage of polysaccharide in EPS and an environment less favorable for microorganisms potentially found in the CA biofilter was proposed. The packing material mineral characteristics do affect polysaccharide implementation in EPS, considering that divalent cations were released with CA.

- A clear difference was noticed in the SEC protein-like fingerprints during biomass enrichment, in terms of both...
Figure 6 Biochemical component proportions of the EPS extracted from RS and CA in different filtration bed layers at Day 360.

Figure 7 Area percentages of PN-like fingerprint fractions of the EPS extracted from RS and CA over the various filtration bed depths.

Figure 8 HS-like HPSEC fingerprints of EPS extracted from RS and CA at various depths on Day 360, and the HS-like fingerprint of feed water.
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Supplementary Figure 2: HPSEC fingerprints of protein-like substances of extracted EPS from filter materials vs. bed thickness.