Evaluation of the extracellular polymeric substances by confocal laser scanning microscopy in conventional activated sludge and advanced membrane bioreactors treating hospital wastewater

Mousaab Alrhmoun, Claire Carrion, Magali Casellas and Christophe Dagot

ABSTRACT

Confocal laser scanning microscopy (CLSM) combined with fluorescent viability indicators, was used in this study to investigate the impact of hospital wastewaters on floc structure and composition. In this work, three pilot-scale projects, two membrane bioreactors (MBRs) with a submerged or external membrane bioreactor and a conventional activated sludge, were installed and operated for 65 days. They were fed with an influent sampled directly from the hospital drainage system, which contained micropollutant concentrations ranging from ng/L to mg/L. Samples of flocs were observed using CLSM to characterize the extracellular polymeric substances (EPS) stained with concanavalin A–tetra methylrhodamine and fluorescein isothiocyanate solution and combined with a fluorescent viability indicator (Baclight® Bacterial Viability Kit, Molecular Probes), allowing visualization of isolated stained cells in the three-dimensional structure of flocs (damaged or not). The results of CLSM of the sludge composition were compared with classical biochemical analysis of EPS made through a thermal extraction method. The results showed a good relation between these analyses and the statistical treatment of microscopic pictures.

Key words | confocal laser scanning microscopy, EPS, extracellular polymeric substances, MBR, membrane bioreactors

Mousaab Alrhmoun (corresponding author)
Magali Casellas
Christophe Dagot
Laboratory of GRESE EA 4330,
University of Limoges,
123 avenues Albert Thomas,
87060 Limoges Cedex,
France
E-mail: *moussab1984@hotmail.com*

Claire Carrion
UMR 7276 CNRS Service commun de microscopie-CIM,
Université de Limoges,
Faculté de Médecine,
Limoges F-87000,
France

INTRODUCTION

In biological wastewater treatment systems, occurrence of extracellular polymeric substances (EPS), a complex high molecular weight mixture of polymers bound to bacterial flocs, has been observed using various electron microscopy techniques (Sheng *et al.* 2010). This often leads to decreased performance of the wastewater treatment plant, to reduce settling properties of activated sludge flocs in the secondary clarifier and, in the worst case, to impact on the water quality (Schmid *et al.* 2003). EPS are composed of a wide variety of organic materials, including polysaccharides, proteins, nucleic acids, lipids and humic substances. In an activated sludge system, EPS mainly contain two forms, soluble EPS (SEPS) and bound EPS (BEPS) according to their location around the cell (Wingender *et al.* 1999; Rosenberger *et al.* 2002).

In recent years, membrane bioreactors (MBRs) have given rise to increased attention in the field of wastewater technologies (Zator *et al.* 2007). An important problem in MBR treatment is the fouling of the membrane and that leads to a decrease in performance of the MBRs. The fouling properties of the membrane can be affected by a number of factors, such as the amount and the composition of EPS, the distribution in the sludge and the structure of flocs. The relative importance of these factors is, however, not well understood (Ramesh *et al.* 2006).

Recently, among the various techniques that exist for the visualization and distribution of EPS in activated sludge or sorbed on the MBR, confocal laser scanning microscopy (CLSM) was found to be a powerful tool used on the micrometre scale (Le-Clech *et al.* 2007; Wang & Wu 2009). CLSM could be used to observe fully hydrated samples to obtain the original shapes and structures of EPS (Zhang & Bishop 2001). After staining by various fluorescence probes, the

doi: 10.2166/wst.2014.141

spatial distributions of carbohydrates, proteins and nucleic acids in EPS can also be obtained by CLSM, in addition to conventional chemical colorimetric analyses which can be used to quantify their contents in EPS (Raunkjær *et al.* 1994).

A comparison between the two methods for carbohydrates, protein and humic-like substances content determination in EPS showed that the two methods yielded similar results, but that the coefficient of time and the analyses cost for the CLSM method was lower than that for the conventional chemical colorimetric method. The purpose of this investigation was to evaluate the suitability of images from the microscopic technique (CLSM) as a basis for quantitative image analysis (Sheng *et al.* 2010).

MATERIALS AND METHODS

Reactor configuration and operating conditions

Three laboratory-scale systems, submerged (SMBR) or external membrane bioreactors (EMBR) equipped with a polypropylene membrane module and a type of hollow fibre membrane, and a conventional activated sludge system (CAS), were used to evaluate the role of the process on the formation of EPS compounds and on the performances of the three configurations in treating hospital wastewater.

Analytical methods

Physico-chemical characteristic measurements on the wastewater and the sludge were carried out every 2 days. Measurements of total and volatile suspended solids (TSS and VSS) were performed according to the normalized method (NF T 90-105; AFNOR 1997). Chemical oxygen demand (COD) was measured by the closed reflux colorimetric method (ISO 15705:2002; ISO 2002), and total nitrogen (TN) was assessed using alkaline persulfate digestion with colorimetric finish (Hach company). The COD and TN measurements were carried out on both total and soluble fractions (after samples had been filtered at 1.2 μm). Ionic species in solution were determined on samples filtered at 0.22 μm using ion chromatography (DIONEX 120) according to the standard method (AFNOR, NF EN ISO 10304-1). The analytical error was ±5%.

EPS extraction and chemical analysis

The analysis of EPS in biomass was made through a thermal extraction method. Protein content was determined according to the method of Lowry *et al.* (1951) and the polysaccharides were determined according to the method of Dubois *et al.* (1956).

Confocal laser scanning microscopy – EPS staining and visualization

SYTO® 9 BacLight™ bacterial stains was used according to the manufacturer's instructions (Molecular Probes, Eugene, Oregon, USA). The kit provides a three-colour fluorescence assay of bacteria relying on membrane integrity: viable bacteria are stained by SYTO® 9 and fluoresce green, while damaged bacteria are stained by propidium iodide and fluoresce red. The protocol established by Lopez *et al.* (2005) and Baker & Inverarity (2004) was performed: 1 mL of undiluted biomass suspension was mixed with 3 μL of a mixture of equal parts of SYTO® 9 and propidium iodide. This short staining protocol allowed direct observation of the original floc structure and the time-lapse microscopy. Microscopic observations started 15 min after staining. Excitation maxima for SYTO® 9 and propidium iodide bound to DNA are 480 and 540 nm, respectively (Reynolds 2002). A Zeiss LCM 710 NLO confocal microscope was used for the image series. The band width of the detected fluorescence wavelengths has been optimized to uniquely channel the maximum emission in sequential mode to avoid potential interference (502–530 nm) for SYTO® 9 and (600–630 nm) for propidium iodide.

Step size was determined by choosing start and end points in the *z*-direction of the flocs, and by then selecting a number of optical sections.

Polysaccharides (PS) and proteins (PN) staining was carried out according to the modified procedure of Chen *et al.* (2007). Bio-samples were centrifuged to remove supernatant, washed twice with phosphate-buffered saline (PBS) buffer (pH 7.2) and kept fully hydrated in 2 mL centrifuge tubes covered with aluminium foil.

For PS staining, 100 μL of concanavalin A conjugated with tetramethylrhodamine (Con A, 250 mg/L; Molecular Probes, Carlsbad, CA, USA) was first dripped onto the sample and incubated for 30 min to stain α-mannopyranosyl and α-glucopyranosyl sugar residues. For PN staining, 100 μL of sodium bicarbonate buffer (0.1 M) was introduced to the sample to maintain the amine groups in non-protonated form. Subsequently, 100 μL of fluorescein isothiocyanate solution (FITC, 1 g/L, Fluka) was supplemented and incubated for 1 h to bind to proteins. Samples were washed twice with 1× PBS buffer after each staining stage to remove loosely bound and excess dyes.

Finally, sectioned granule or biofloc samples were mounted onto microscope glass slides for observation of the distribution of PS and PN by CLSM. The image acquisition settings, such as laser intensity, numerical aperture, gain and offset settings were adjusted according to Toh *et al.* (2003). Samples were visualized with 10× and 100× objectives and analyzed with the start LSM image browser confocal and Image J software.

Digital image analysis

Image analysis was performed with the freely available software Image J version 1.39i including the LSM-Reader plug. The tool Image J Analyzer 1.1, which is based on the performance of Image J and handles LSM5 formatted image stacks, was programmed for quantitative analysis. By setting a threshold, pixels with intensity below the threshold were assigned to the background. All other pixels were set to the foreground. Due to the individual image adjustment during the image stack acquisition, the threshold was chosen manually for each image stack. It has to be stressed that the pitfalls of threshold setting by the operator are well known (Yang *et al.* 2001; Staudt *et al.* 2004).

Table 1 | Stabilized COD, N and SS removal efficiencies for AG-MBR and SG-MBR

Efficiency of removal (%)	TSS	VSS	Total COD	N	Soluble COD	N
EMBR	99.6	97.5	87.9	91.1	86.9	90.5
SMBR	93.6	87.8	80.5	79.0	71.6	85.4
CAS	93.4	87.2	78.0	85.4	72.8	85.5

RESULTS AND DISCUSSION

Performance of MBR systems and CAS in treating the organic pollutants

A comparison between the three reactors was made for TSS and VSS, COD and nitrogen removal rates. The results in Table 1 showed that the removal efficiencies of COD, TN, and TSS, in EMBR were 87.9, 91.1 and 99.6%, respectively, compared with 80.5, 79 and 93.6%, respectively, in SMBR and 78, 85.4 and 93.4% in CAS (Table 1). The MBR system is able to achieve COD removal by both physical and biological mechanisms.

Analysis of EPS

It has been generally believed that EPS can mediate both bacterial cohesion and adhesion. Hence, EPS, especially PS/PN, have a decisive role in building and keeping the structural integrity of a microbial community (Liu *et al.* 2004). At low solid residence time (SRT) (15 days), the concentrations of all the compounds measured in MBR sludge supernatant are higher than those with CAS until 45 days of exposure (Figure 1). These results could be explained by different assumptions: (1) first it could be assumed that a 'low' SRT (lower than 30 days) is not sufficient to degrade

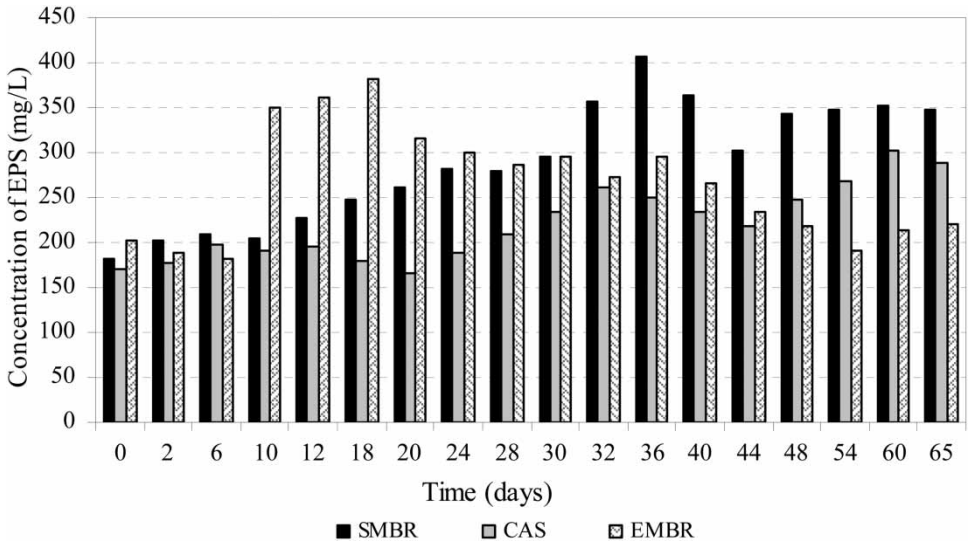


Figure 1 | EPS concentration in the mixed liquor for SMBR, EMBR and CAS systems.

all the organic compounds, which are accumulated in the system because of external membrane retention; (2) the presence of more dispersed organisms in MBR probably also aid the degradation of molecules in the supernatant due to reduction of mass transfer limitation; (3) increase of SRT could also enhance the development of slowly growing populations, which are able to use some macro-molecules (polysaccharides and proteins) as substrate; (4) finally, if it is assumed that the quantified organics (PN and PS) are principally composed of microbial products, it can be supposed that non-flocculating bacteria produced less biopolymer, which is a known flocculating agent.

EPS analysis with confocal laser scanning microscopy

Live/dead assessment within mixed microbial flocs

Propidium iodide (PPI) was used to stain dead cells and extracellular SYTO® 9 was used to stain live cells in the sample. In general, for all floc samples, the SYTO® 9 and PPI signals were distributed throughout the flocs sections. However, the intensity of the two signals varied from one region of a floc to another, probably due to the differential number and localization of live versus dead cells within the flocs.

CLSM images (Figure 2) reveal that there is an increase in blue signal intensity during the time of observation and it is much more significant in the SMBR compared with the CAS. These modifications were attributed to a protection mechanism of the bacteria against toxic effluent (Avella *et al.* 2010). EPS might act as protective barriers against toxic substances, e.g. heavy metals or certain biocides (disinfectants and antibiotics).

Stacks of images were imported into the image software to mathematically compute relative intensities of the SYTO® 9 and PPI signals, and to calculate the relative percentage of live cells per floc.

Evolution of EPS compositions within a mixed microbial community

Figures 3 and 4 show that the visualizations of flocs collected in the EMBR reactor after 2, 20, 45, and 65 days of exposure time to the hospital effluent.

In general, through visual inspection, an increase in SEPS and BEPS was observed, especially in the first days between 2 and 20 days of exposure. This finding is in accordance with our chemical analysis which shows increasing concentration of SEPS and BEPS (PS, PN and humic-like substances) with time up to 20 days (see Figures 1, 3 and 4). Through the fluorescence indications at the start of the study (from 2 to 20 days of exposure to hospital effluents) it were observed that the polysaccharides and the humic-like substance compounds

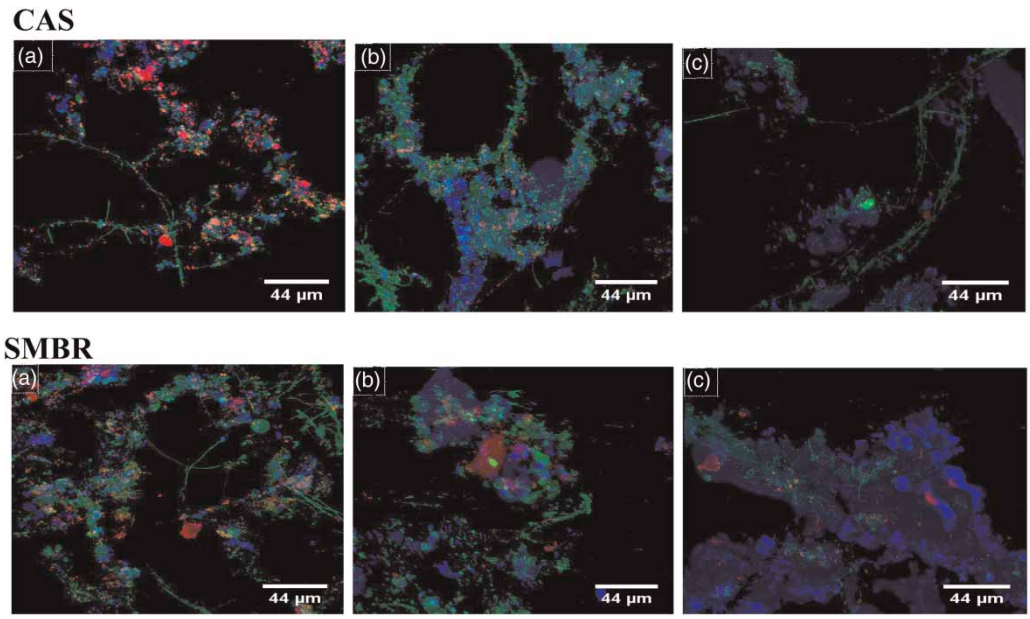
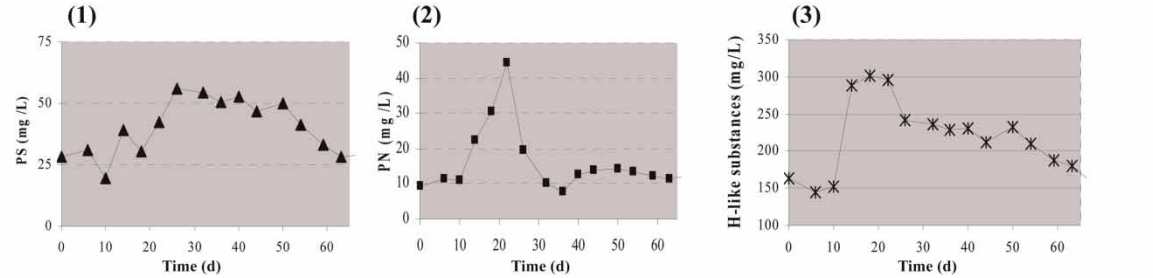
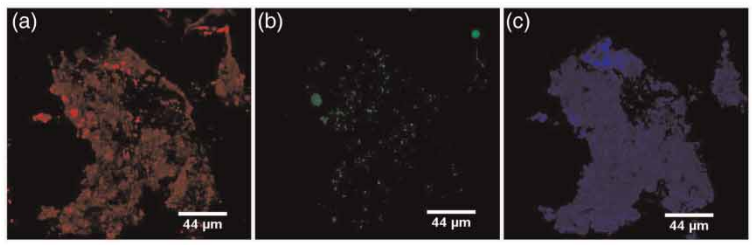
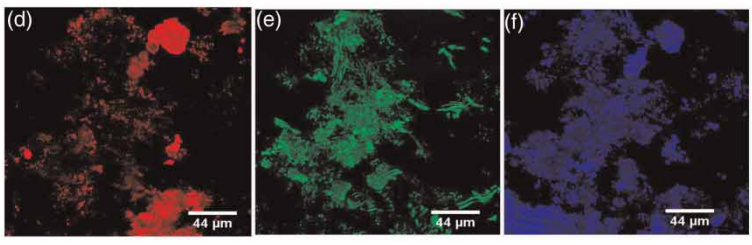


Figure 2 | CLSM image of live cell distribution within CAS and SMBR flocs. Flocs were stained with SYTO® 9 for total available DNA (viable bacteria; green) and stained with PPI for DNA of dead cells and EPS DNA (dying bacteria; red). Images obtained at x100 magnification. These representative images are based upon the examination of 5–10 flocs per sample. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.

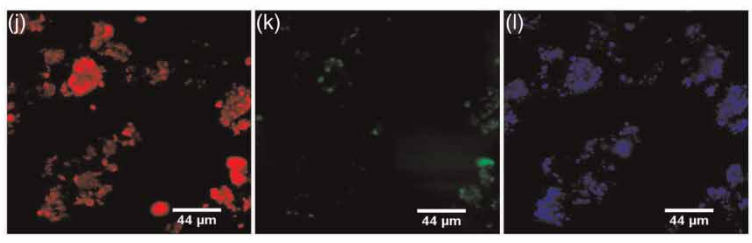




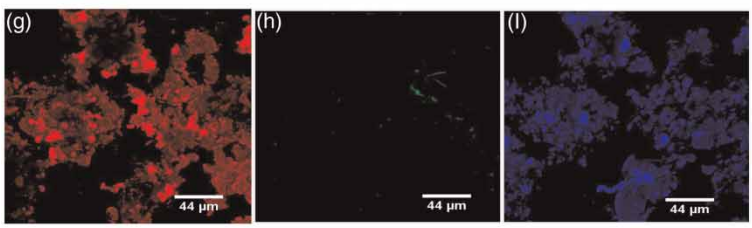
Label	Area	Mean
Red	50625.003	19.702
Green	50625.003	1.247
Blue	50625.003	27.053
(R+G+B)/3	50625.003	16.000
0.299R+ 0.587G+0.114B	50625.003	9.705



Label	Area	Mean
Red	50625.003	23.213
Green	50625.003	31.392
Blue	50625.003	49.012
(R+G+B)/3	50625.003	34.535
0.299R+ 0.587G+0.114B	50625.003	30.964



Label	Area	Mean
Red	50625.003	46.362
Green	50625.003	0.408
Blue	50625.003	35.379
(R+G+B)/3	50625.003	27.382
0.299R+ 0.587G+0.114B	50625.003	18.133

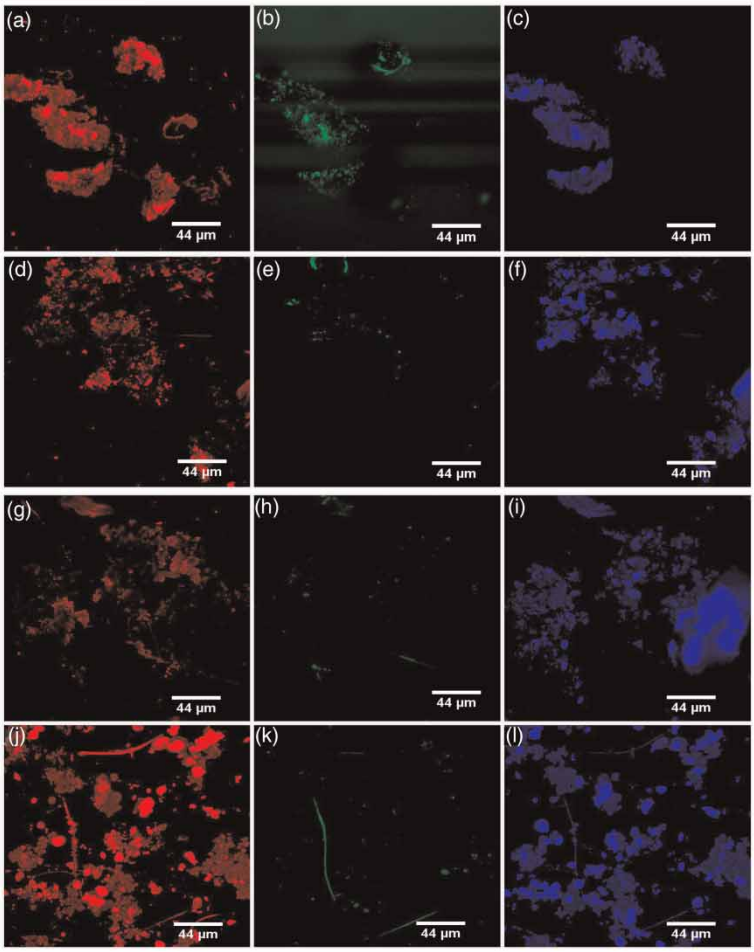


Label	Area	Mean
Red	50625.003	18.115
Green	50625.003	2.982
Blue	50625.003	13.466
(R+G+B)/3	50625.003	11.516
0.299R+ 0.587G+0.114B	50625.003	8.710

Figure 3 | CLSM images of the BEPS distribution within EMBR flocs. Images were obtained at x10 magnification. FITC staining universal protein is in green and ConA staining α-mannopyranosyl and α glucopyranosyl is in red. Images are representative of 5–10 flocs examined. Images (a, b, c), (d, e, f), (g, h, i), (j, k, l) are for 2, 20, 45 and 65 days, respectively. (1), (2), (3) represent the distribution of the EPS constituent versus the time in the sludge from the experimental tests. In right-hand boxes, 'Red' denotes polysaccharides, 'Green' denotes proteins, and 'Blue' denotes humic-like substances. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.

increased in the bound phase (Figure 3). Then, a stabilization in polysaccharides concentration and a decrease in concentration of proteins were observed in both phases (bound and soluble) of sludge samples after more than 40 days.

The chemical analysis of the soluble and supernatant EPS matrix in the EMBR shows a slight trend towards a small increase in carbohydrate concentrations and a decrease in protein concentrations after the first 20 days of



Label	Area	Mean
Red	50625.003	17.822
Green	50625.003	17.301
Blue	50625.003	9.458
(R+G+B)/3	50625.003	14.856
0.299R+ 0.587G+0.114B	50625.003	16.583

Label	Area	Mean
Red	50625.003	12.017
Green	50625.003	0.621
Blue	50625.003	16.426
(R+G+B)/3	50625.003	9.686
0.299R+ 0.587G+0.114B	50625.003	5.829

Label	Area	Mean
Red	50625.003	8.130
Green	50625.003	0.542
Blue	50625.003	27.557
(R+G+B)/3	50625.003	12.075
0.299R+ 0.587G+0.114B	50625.003	5.886

Label	Area	Mean
Red	50625.003	43.447
Green	50625.003	76.256
Blue	50625.003	30.918
(R+G+B)/3	50625.003	50.208
0.299R+ 0.587G+0.114B	50625.003	61.277

Figure 4 | CLSM images of the SEPS distribution within EMBR flocs. Images were obtained at x10 magnification. FITC staining universal protein is in green and ConA staining α-mannopyranosyl and α glucopyranosyl is in red. Images are representative of 5–10 flocs examined. Images (a, b, c), (d, e, f), (g, h, i), (j, k, l) are for 2, 20, 45 and 65 days, respectively. In right-hand boxes, 'Red' denotes polysaccharides, 'Green' denotes proteins, and 'Blue' denotes humic-like substances. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.

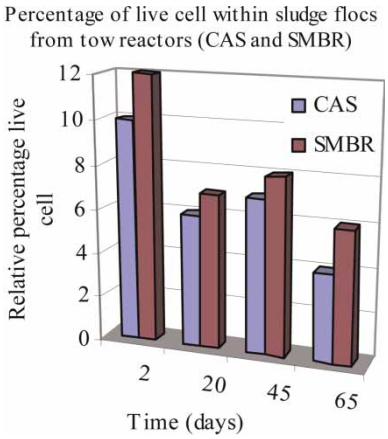


Figure 5 | Relative number of live cells in two reactors (CAS and SMBR). The percentage was determined by measuring fluorescent intensities of SYTO® 9, which labels all DNA in a sample, and PPI, which labels DNA from cells with compromized membranes and extracellular DNA. The calculation is based on measurements of 5–10 flocs from each sampling site.

the study. The lower signal intensity at the exterior of the flocs (soluble) compared to the interior of flocs (bound) can be related to the loss of packing of EPS.

A dynamic high and low signal observed on the two sides of the central peak was possibly due to the porosity and filamentous bacteria on the exterior of the flocs. Lower intensities in the FITC could be due to fewer binding sites caused by lower concentration of these EPS constituents.

Statistical analyses

Stacks of images were imported into the J image software to mathematically compute relative intensities of the SYTO® 9 and PPI signals, and to calculate the relative percentage of live cells per floc. The relative distribution of dead/live

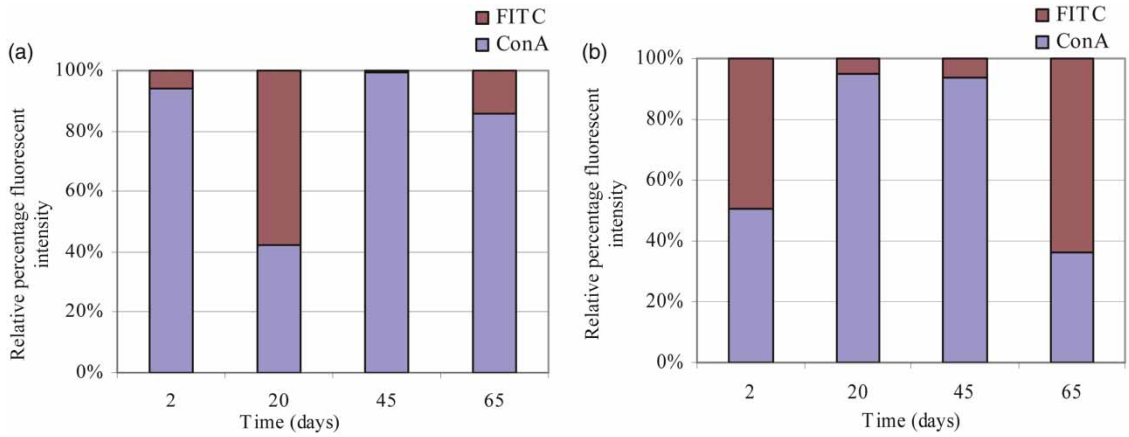


Figure 6 | Percentage of protein and carbohydrate intensity versus the time within EMBR flocs for the bound phase (a) and soluble phase (b) ($n = 5$).

cells is shown in Figure 2 and relative percentages can be observed in Figure 5 for two sites.

Five or more flocs from each sampling site were examined. We can observe the SMBR samples had the most intact cells, followed by CAS. This may indicate the overall health and well-being of cells within flocs of different origin and give an indicator on the relation between concentration of EPS and the live cells of bacteria.

The settleability and physicochemical parameters are attributed to the PN/PS ratio. The PN/PS ratios were analyzed via the bulk extraction method for the sludge samples in both phases (bound and soluble) in EMBR and was found to be from 0.3 to 1.3 in both phases.

For the sludge samples analyzed through microscopic analysis, however, the calculated PN/PS ratios were between 0.05 and 1.3 at the bound phase.

The bulk analysis revealed that the bulk EPS constituents of all sampling sites were not significantly different according to PN/PS ratios examined through the microscopic method. The relation between the intensity of the PN (FITC) and the PS (Con A) with the relative percentage fluorescent intensity versus time is reported in Figure 6.

CONCLUSIONS

It can be concluded that CLSM, in combination with image analysis, is a powerful method for direct determination of the EPS distribution, heterogeneity factors and the structure of activated sludge flocs. We have also found that there is a good correlation between the chemical analyses of EPS and the statistical treatment of microscopic pictures.

In addition, significant and specific changes of the EPS compounds, and in the microbial population structure of original flocs, could be observed in the CAS and MBR systems treating hospital effluent.

ACKNOWLEDGEMENTS

This work was supported by the noPILLS project (www.no-pills.eu), Department of Rural Engineering at University of Aleppo. The authors are thankful to David Chaisemartin for technical assistance.

REFERENCES

AFNOR 1997 NF T90-105 Qualité de l'eau – Dosage des matières en suspension – Méthode par centrifugation. AFNOR, Paris.

Avella, A. C., Delgado, L. F., Gorner, T., Albasi, C., Galmiche, M. & De Donato, P. h. 2010 Effect of Cytostatic drug presence on extracellular polymeric substances formation in municipal wastewater treated by membrane bioreactor. *Bioresource Technology* **101**, 518–526.

Baker, A. & Inverarity, R. 2004 Protein-like fluorescence intensity as a possible tool for determining river water quality. *Hydrology Processes* **18**, 2927–2945.

Chen, M., Lee, D. J. & Tay, J. H. 2007 Distribution of extracellular polymeric substances in aerobic granules. *Applied Microbiology Biotechnology* **73**, 1463–1469.

Dubois, M., Gilles, K. A., Hamilton, J. K., Robers, P. A. & Smith, F. 1956 Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28** (3), 350–356.

ISO 2002 15705: 2002 Water quality – Determination of the chemical oxygen demand index (ST-COD) – Small-scale sealed-tube method. ISO, Geneva.

Le-Clech, P., Marselina, Y., Ye, Y., Stuetz, R. M. & Chen, V. 2007 Visualisation of polysaccharide fouling on microporous membrane using different characterisation techniques. *Journal of Membrane Science* **290**, 36–45.

Liu, Y., Yang, S. F. & Tay, J. H. 2004 Improved stability of aerobic granules by selecting slow-growing nitrifying bacteria. *Biotechnology Journal* **108**, 161–169.

Lopez, C., Pons, M. N. & Morgenroth, E. 2005 Evaluation of microscopic techniques (epifluorescence microscopy CLSM, TPE-LSM) as a basis for the quantitative image analysis of activated sludge. *Water Research* **39**, 456–468.

Lowry, O. H., Roserbrough, N. J., Farr, A. L. & Randall, R. J. 1951 Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** (1), 265–275.

Ramesh, A., Lee, D. J., Wang, M. L., Hsu, J. P., Juang, R. S., Hwang, K. J., Liu, J. C. & Tseng, S. J. 2006 Biofouling in membrane bioreactor. *Separation Science and Technology* **41**, 1345–1370.

Raunkjær, K., Hvitved-Jacobsen, T. & Nielsen, P. H. 1994 Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. *Water Research* **28**, 251–261.

Reynolds, D. M. 2002 The differentiation of biodegradable and non-biodegradable dissolved organic matter in waste waters using fluorescence spectroscopy. *Journal Chemical Technology and Biotechnology* **77**, 965–972.

Rosenberger, S., Evenblij, H., te Poele, S., Wintgens, T. & Laabs, C. 2002 The importance of liquid phase analyses to understand fouling in membrane assisted activated sludge processes – six case studies of different European research groups, M. Kraume, Filterability of activated sludge in membrane bioreactors. *Desalination* **146**, 373–379.

Schmid, M., Thill, A., Purkhold, U., Walcher, M., Bottero, J. Y., Ginestet, P. h., Nielsen, P. H., Wuertz, S. & Wagner, M. 2003 Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis. *Water Research* **37**, 2043–2052.

Sheng, Y. H., Li, X. & Ping, G. 2010 Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems. A review. *Biotechnology Advances* **12**, 882–894.

Staudt, C., Horn, H., Hempel, D. C. & Neu, T. R. 2004 Volumetric measurements of bacterial cells, extracellular polymeric substance glycoconjugates in biofilms. *Biotechnology and Bioengineering* **88**, 585–592.

Toh, S. K., Tay, J. H., Moy, B., Ivanov, V. & Tay, S. L. 2003 Size effect on the physical characteristics of the aerobic granule in a SBR. *Applied Microbiology Biotechnology* **60**, 687–695.

Wang, Z. & Wu, C. 2009 A review of membrane fouling in MBRs: characteristics and role of sludge cake formed on membrane surfaces. *Separation Science and Technology* **44**, 3571–3596.

Wingender, J., Neu, T. R. & Flemming, H. C. 1999 *Microbial Extracellular Polymeric Substances: Characterization, Structure, and Function*. Springer, Berlin.

Yang, X., Beyenal, H., Harkin, G. & Lewandowski, Z. 2001 Evaluation of biofilm image thresholding methods. *Water Research* **35**, 1149–1158.

Zator, M., Lopez, F. & Guell, C. 2007 Membrane fouling characterization by confocal microscopy during filtration of BSA/dextran mixtures. *Journal of Membrane Science* **301**, 57–66.

Zhang, X. Q. & Bishop, P. L. 2001 Spatial distribution of extracellular polymeric substances in biofilms. *Journal of Environment and Engineering* **127**, 850–860.

First received 11 October 2013; accepted in revised form 4 March 2014. Available online 26 March 2014