Membrane biofouling mechanism in an aerobic granular reactor degrading 4-chlorophenol

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ABSTRACT

The membrane fouling of an aerobic granular reactor coupled with a submerged membrane in a sequencing batch reactor (SBR) was evaluated. The fouling analysis was performed by applying microscopy techniques to determine the morphology and structure of the fouling layer on a polyvinylidene fluoride membrane. It was found that the main cause of fouling was the polysaccharide adsorption on the membrane surface, followed by the growth of microorganisms to form a biofilm.

Key words | 4-chlorophenol, aerobic granules, fouling, membrane, SBR

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INTRODUCTION

Aerobic granules are aggregates of microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than activated sludge flocs (de Kreuk *et al.* 2007). Aerobic granules in an aerobic sequencing batch reactors have several advantages compared to conventional activated sludge in the case of toxic compounds degradation Arrojo *et al.* 2004). It has been proposed that the combination of aerobic granular biomass and a submerged membrane bioreactor (MBR) (Li *et al.* 2005) reduce the membrane fouling by taking advantage of the physical properties of the granules. Previously, there have been reports that state that, although the fouling is delayed when granular sludge is used, a higher irreversible fouling occurs than when conventional activated sludge is used (Wang *et al.* 2008), resulting in a lack of total recovery of the membrane permeability after chemical washing (Zhou *et al.* 2007). Juang *et al.* (2010a, 2010b), reported the presence of an internal bacterial biofilm as a possible factor responsible for the fouling of hollow-fiber membranes.

The fouling of the membranes in a submerged MBR is principally caused by the deposition of extracellular polymeric substances (EPS), together with the biomass forming bio-cake which blocks the filtering (Chen *et al.* 2006a). These exopolymers are formed by proteins and polysaccharides as well as by humic substances, lipids and nucleic acids, in a lower concentration. However, it is unclear about the role played by these substances in the development of the fouling layer throughout time. To determine the biofouling mechanism it is necessary to determine the presence of the different substances, such as EPS, on the membrane surface and pores. This analysis can be done by applying some microscopy techniques (Chen *et al.* 2007). In this sense, the use of fluorescence microscopy and confocal laser scanning microscopy (CLSM) as tools to analyze the structures and bacterial biofilms formation and the presence of exopolymeric substances in bio-cakes on membrane surfaces is well recognized.

The fluorophores used in microscopy analysis are fluorescent molecules that possess reactive groups which covalently and specifically bind to compounds of interest (e.g. isothiocyanate esters, and pentafluorophenyl succinimidyl present in certain fluorochromes) and react with the amino groups contained in some macromolecules (Adav *et al.* 2010). Different fluorochromes, with different excitation wavelengths, can be used to stain the total cells, dead cells, proteins, lipids, and EPS in bioaggregates. Using staining techniques, Chen *el al.* (2006b) found that β-polysaccharides are mostly present near to the membrane

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surface while proteins and α-polysaccharides are located and uniformly distributed in the fouling cake.

To the best of our knowledge, there is no generally accepted mechanism for the fouling process in membrane aerobic granular sludge systems. Also, the role of microorganisms and EPS in different stages of membrane fouling is still unclear. The purpose of this work was to characterize the presence of proteins and polysaccharides throughout the fouling process of a granular sludge system coupled to a submerged membrane, degrading an inhibitory compound (4-chlorophenol), by using microscopy methods.

MATERIAL AND METHODS

Experimental set-up

A membrane sequencing batch reactor (MSBR) with a total volume of 5 L (4 L of working volume) was used. The reactor had an internal diameter of 9.5 cm. The MSBR was inoculated with aerobic granules acclimatized to degrade 200 mg/L of 4-chlorophenol (4CP), harvested from a reactor operated with a hydraulic retention time (HRT) of 8 h (Arellano-Badillo *et al.* in press). The reactor was fed with synthetic wastewater containing 200 ± 15 mg4CP/L (Sigma-Aldrich, USA), as a sole source of carbon and energy, and oligoelements (AFNOR 1985). The chemical oxygen demand (COD)/N ratio in the reactor was adjusted to 35 and maintained by adding NH_4Cl (Baker, USA). The initial concentration of 4CP in the reactor was maintained constant during all the study.

The MSBR was equipped with an oxygen sensor (Endress-Hauser, Germany). A fine bubble diffuser was placed at the bottom of the reactor to provide 60 L of air per hour, which provided an air surface velocity of 0.235 cm/s. The reactor temperature was maintained at 20 ± 1 °C using temperature controlled re-circulated water in an external double-wall at the reactor. pH was maintained at 7.0 ± 1, by the addition of 0.1 N NaOH or H_2SO_4. For the filtration period, two modules with four tubular membranes (10 mm diameter) each made of polyvinylidene fluoride (Memos GMBH, Germany) were used (Figure 1). The membranes had a molecular weight cut-off of 250 kDa and a total area of 0.08 m^2. Permeate suction was supplied using a peristaltic pump (Cole Palmer, Masterfelx). The pumping line was coupled to a pressure transducer (C206, Cole Palmer, Masterflex). The method for backwashing proposed by Vargas *et al.* (2008) was used to reduce the reversible fouling. For the backwashing period (15 sec in every 15 min of operation at a flow rate of 10.4 L/h) a diaphragm pump (Concept Plus Prominent) was used. Aeration was maintained during filtration. In order to maintain 1.5 g/L of volatile suspended solids (VSS), the reactor was purged between day 15 and 25. The solids retention time, SRT, was maintained at 40 d.

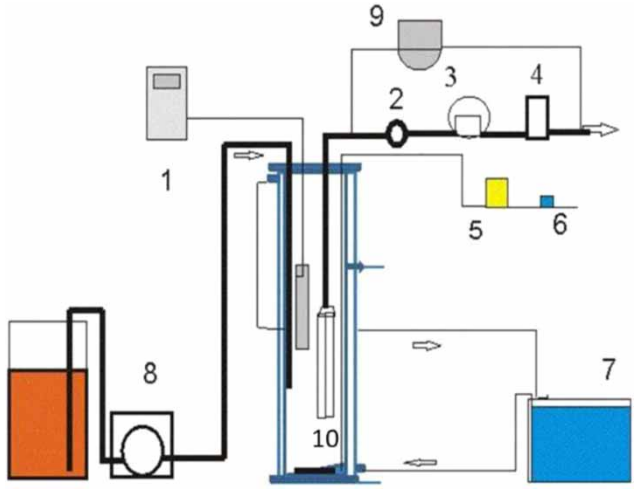

Figure 1 | Schematic set-up of the membrane sequencing batch reactor. (1) Oxygen meter; (2) pressure gauge; (3) pump; (4) flow meter; (5) mass flow controller; (6) air-supplying valve; (7) water temperature device; (8) feeding pump; (9) backwashing pump; (10) membrane.

The MSBR was operated in the following schedule: filling (6 min), reaction (varied from 140 to 210 min depending on the time needed to remove >99% of 4CP), filtration (varied from 100 to 720 min depending on the time needed to draw off 2 L of treated water). The MSBR was operated with an exchange volume of 50%. No chemical cleaning was applied since the purpose of the study was to evaluate the fouling process over time.

Analytical methods

The 4CP concentration was measured as total phenols by collecting samples and processing them offline with a colorimetric technique using 4-aminoantipyrine (APHA 1992). Total suspended solids and VSS were determined according to *Standard Methods* (APHA 1992). Dissolved organic carbon was determined with a Shimadzu TOC-5050 analyzer (Japan). COD, sludge volumetric index (SVI) and settling velocity were evaluated according to *Standard Methods* (APHA 1992). EPS were calculated as the sum of the protein and polysaccharide concentrations according to Li *et al.* (2008). EPS are classified into two types: free EPS, that are able to move freely between sludge flocs and the surrounding liquor, and bound EPS that are the EPS bound tightly to solid surfaces (Wingender *et al.* 1999). Bound and free EPS were analyzed

according to the method described by Arellano-Badillo *et al.* (in press). Briefly, the free EPS were determined by centrifuging a sample of mixed liquor and the supernatant was filtered through a glass fiber filter; then, the filtrate was used to measure free polysaccharides. The bound EPS were obtained after heat treatment of the sample, and then by applying a similar procedure as for free EPS. Protein and polysaccharides were measured by Lowry and Dubois methods, respectively. Bovine serum albumin and glucose (Sigma, USA) were used as standards for the protein and polysaccharide analyses, respectively.

The percentage of granules with a diameter higher than 210 μm was evaluated by granulometry according to Laguna *et al.* (1999). The granular biomass was sieved in a standard sieve of 0.21 mm (Tyler's number 65), based on the definition of de Kreuk *et al.* (2007). The evolution of granule size over time was determined with 0.5-mL samples using an optical microscope (DM500, Leica, Japan); the images were processed with the AxioVision Rel 4.8.2, Carl Zeiss software. For each sampling, 10 measurements were performed, and the mean was calculated. The data represent the median of 10-sample mean values for each sampling time.

Morphology and evolution of fouling

In order to study the membrane fouling, CLSM microscopy techniques were applied to analyze the membrane samples (after 10, 15, 40 and 60 days of operation without chemical cleaning). Autopsies of the submerged membranes were performed as follows. The membrane was removed from the reactor, and approximately one centimeter was cut and placed in a polyethylene tube. The sample was fixed as recommended by Adav *et al.* (2010). Transverse and horizontal cuts were performed by a cryotome (CM 1850, Leica, Japan), using optimal cutting temperature compound (Sakura Finetek, USA) as mounting medium. Transversal cuts were made of 40 microns thickness. Samples were stained by applying different fluorochromes. First 100 μL of sodium bicarbonate buffer (0.1 M, pH = 9) was added carefully, in order to maintain the protein amino group of the bioaggregate in its unprotonated form. Then, 10 μL of a solution of isothiocyanate fluorescein (staining proteins) and 1 mg/mL of dimethylsulfoxide were added, and stirred by an orbital shaker at 100 rpm for 30 min. Following incubation, the fluorochrome calcofluor white (100 μL, 30 mg/L in 1× phosphate-buffered saline (PBS), pH = 7.2 for 30 min) was added to stain polysaccharides (Adav *et al.* 2010). Prior to observation, the sample was stained with ToPro 3 (250 mg/L in PBS, pH = 7.2) for 15 min, to visualize dead cells (Adav *et al.* 2010). The remaining dye was then removed by washing twice with 1× PBS buffer. Samples were analysed in a CLSM microscope (Axiovert 200 LSM 510 Meta detector with multiphoton laser, Zeiss, Germany).

To evaluate the fouling morphology, scanning electron microscopy (SEM) was also used, according to the methodology proposed by Ng *et al.* (2006). The sample was coated with a gold layer in a sputter coater and observed under an EVO 50 microscope (Zeiss, Germany). In addition, transmission electron microscopy (TEM) was used to observe details of the morphology of microorganisms (JEOL JEM-1010, Japan). For TEM analysis, the sample was fixed in 2% (v/v) glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. The sample was washed three times (15 min each) with the same cacodylate buffer with 8.5% saccharose. The sample was fixed with a 1% osmium solution in 0.1 M cacodylate buffer for 1 h at 4 °C. Membrane cuts were made once at the end of the reaction time in order to avoid interferences with the substrate. Dehydration was performed with ethanol with gradually increasing concentrations, as described by Lemaire *et al.* (2008). The processed sample was mounted in resin and a cut of 60 nm was made. For SEM and TEM analysis, samples were taken at 10, 15, 40 and 60 days.

RESULTS AND DISCUSSION

Reactor operation

The MSBR reactor was operated for a period of 80 days with the aerobic granules. During the operation of the MSBR, the degradation efficiencies achieved were 97.5% ± 1.1% as COD removal and 99.9% ± 0.1% as 4CP removal. The membrane modules used were not chemically cleaned throughout the operation, in order to evaluate the progression of the fouling process. During the first 25 days of the reactor operation, a decrease in the amount of suspended solids was observed.

The reactor was inoculated with an initial VSS concentration of 2 g/L. In order to maintain 1.5 g/L of VSS, the reactor was purged between day 15 and 25. Then, the VSS concentration was stabilized at 1.46 ± 0.46 g/L (Figure 2(a)). This VSS concentration is typical for aerobic granules formed in recalcitrant wastewaters since bacteria show a low growth rate (Carucci *et al.* 2009; Khan *et al.* 2011). In the case of 4CP, the observed yield, in terms of suspended solids, was 0.3 mg VSS/mg 4CP, which explains the low biomass production (Moreno-Andrade & Buitrón 2004). The percentage of aerobic granules greater than 210 μm, was 78.6 ± 16.4% and their diameters were maintained at 2.46 ± 0.16 mm. The

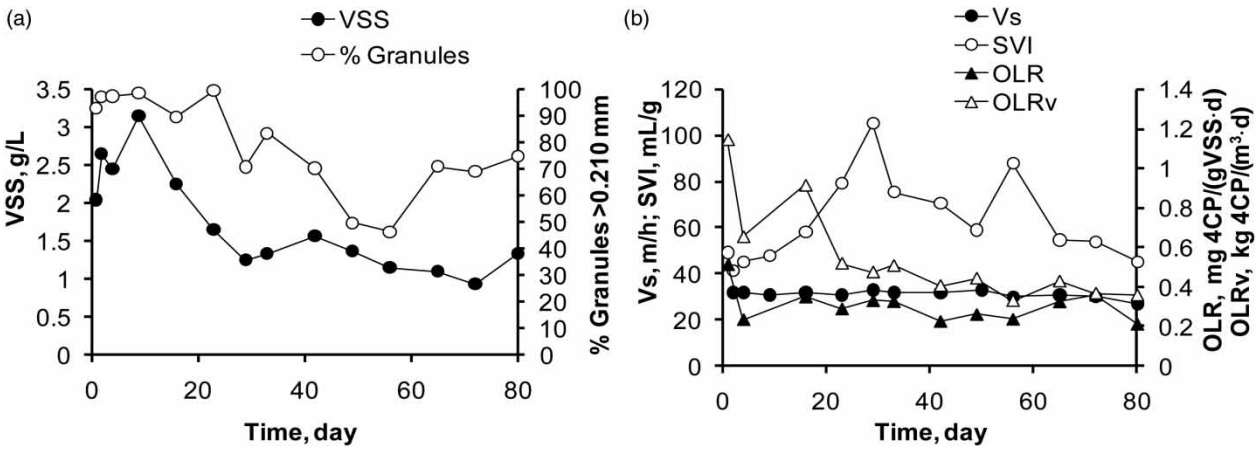

Figure 2 | (a) Variation in biomass concentration and granulation percentage and (b) sludge volumetric index (SVI), settling velocity (Vs), volumetric organic loading rate (OLRv) and organic loading rate (OLR) during the operation of the MSBR.

SVI was 61 ± 16 mL/g and the Vs was 29.9 ± 2.7 m/h demonstrating excellent settling characteristics of the aerobic granules (Figure 2(b)).

Figure 3 shows the total (Figure 3(b)) and free EPS (Figure 3(b)) evaluated during the reactor operation. Not much variation in EPS content for the biomass and supernatant (total) as well as in the supernatant (free) was observed. Thus the variation of the VSS did not influence the EPS content.

The degradation of 4CP was completed in 2.5 ± 0.7 h; however, as the fouling started, the time required to draw the reactor increased, and consequently the time required to complete the total cycle increased from 4 h (on day 1), to 13 h (on day 50) (Figure 4(a)). Reaction time was

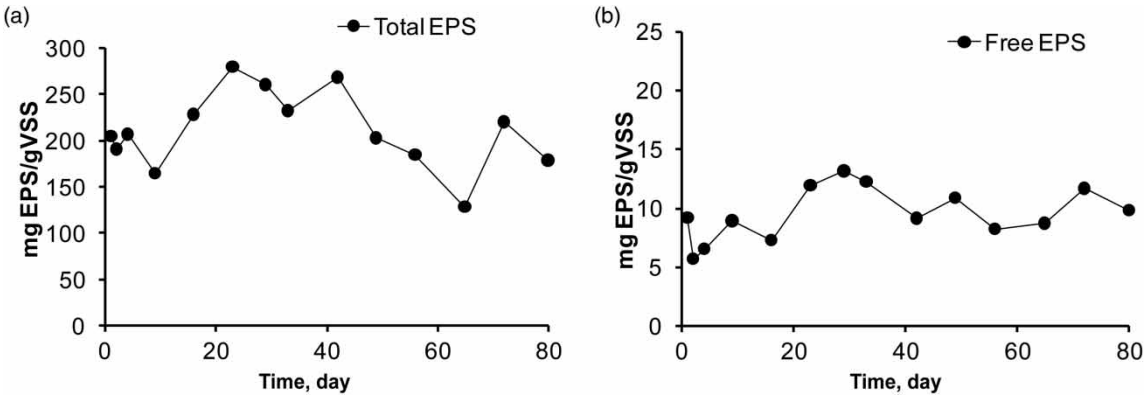

Figure 3 | Total (a) and free (b) EPS obtained during the membrane operation.

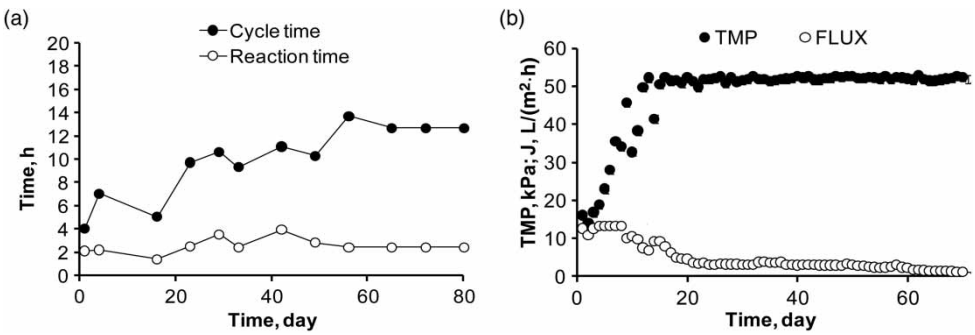

Figure 4 | (a) Variation in cycle duration due to membrane fouling (note that reaction time was maintained constant) and (b) variation in transmembrane pressure (TMP) and flux during operation of the MSBR.

fixed, but due to the membrane fouling, the HRT was increased in order to filter the same amount of water as shown in Figure 4(a). After 15 days, the transmembrane pressure (TMP) increased to values greater than 50 kPa and the flux decreased from 12.5 L/(m^2·h) (day 1) to 2.8 L/(m^2·h) (day 25) and 0.7 L/(m^2·h) (day 80) (Figure 4(b)). The backwash was automatically controlled and the system performed backwashing when it reached a TMP of 55 kPa in order to protect the integrity of the membrane. The fouling of the membrane increased the frequency of backwashing. In order to evaluate the dynamics of fouling, autopsies of the membrane were performed at 10, 18, 40 and 60 days of operation.

Evaluation of the fouling mechanism

TMP indicated that fouling had occurred by 10 days of operation. CLSM analysis showed, at this period, an almost uniform deposit of polysaccharides (gray color in Figure 5(b); blue in online version) on the membrane surface and interior. Microorganisms at the surface began to produce proteins to form aggregates (white color in Figure 5(a); green in online version). At day 40, there was a significant presence of microorganisms, including bacteria, embedded in a matrix composed mainly of proteins (Figure 5(a), superficial). Some polysaccharides were found in the interior of the membrane (Figure 5(b), transversal cut). The biofilm was not

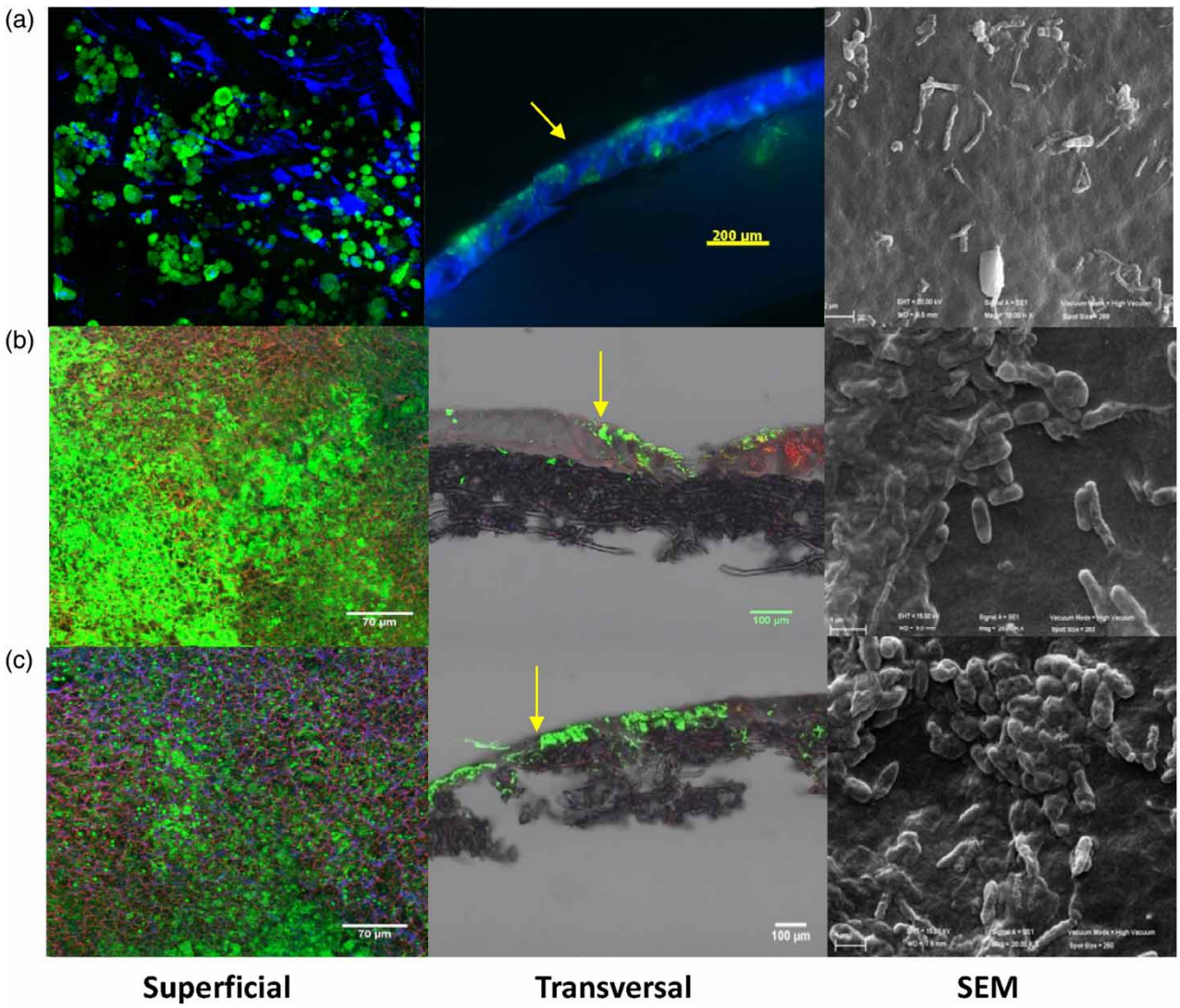

Figure 5 | CLSM images of superficial and transversal sections of the membranes and superficial SEM images at (a) 10 d, (b) 40 d and (c) 60 d. For the superficial and transversal sections, white = proteins (green in online version), and blue = polysaccharides (blue in online version). Red = bacteria cells. Arrows indicate the membrane surface. The full color version of this figure is available online at http://www.iwaponline.com/wst/toc.htm.

uniform across the surface (Figure 5(b), SEM), but formed bulky structures and channels, which is typical for mature biofilm matrices (Lemaire *et al.* 2008). At day 60 (Figure 5(c)), less biomass was observed on the membrane surface. However, the membrane was totally clogged by the polysaccharides deposited on the surface. Here a large amount of protein aggregates were also observed. Their appearance is more compact than observed on the previous sampling days, which could be related to the SRT of 40 days and a high hydraulic residence time of 28 hours maintained in this study, which has also been reported previously by Lee *et al.* (2003). At this time, a biofilm detachment was observed in some areas of the membrane surface.

The SEM and TEM images allowed us to spatially identify the microorganisms which are colonizing the surface of the membrane (Figures 6 and 7). At day 15, the SEM images showed bacillus-shaped bacteria (0.3 to 0.5 μm wide, 1 to 2 μm long) with EPS adhered to the membrane surface in a monolayer (Figure 6(a)). Some attached granular formations including protozoa and remaining EPS (Figure 6(b)) were also observed. These ciliate protozoa (*Opercularia*) have also been observed on the surface of

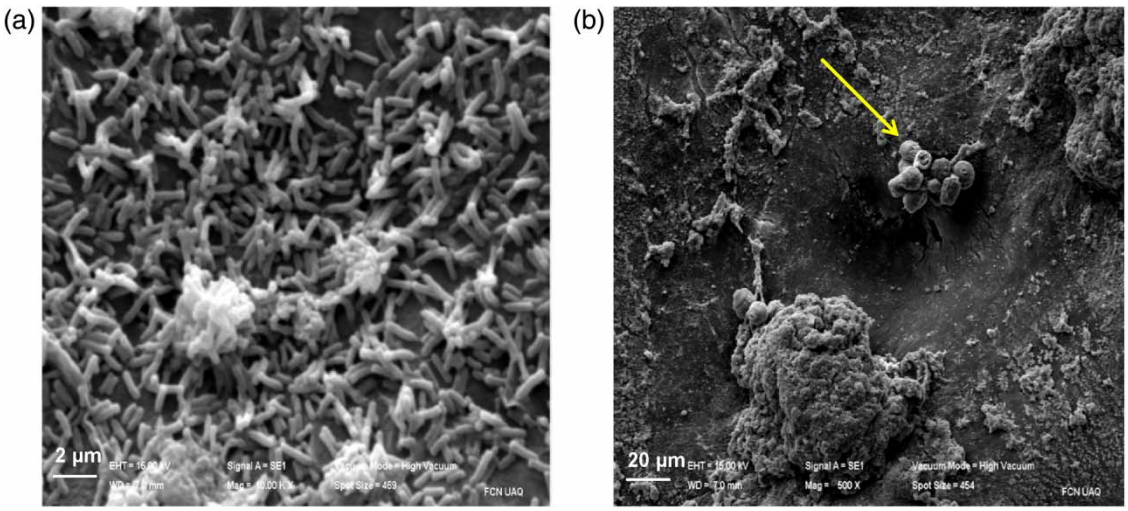

Figure 6 | SEM micrographs at day 15. (a) Superficial fouling biofilm (monolayer); (b) bacterial clusters (granular-kind) and protozoa (arrow) growing on the biofilm bacterial monolayer.

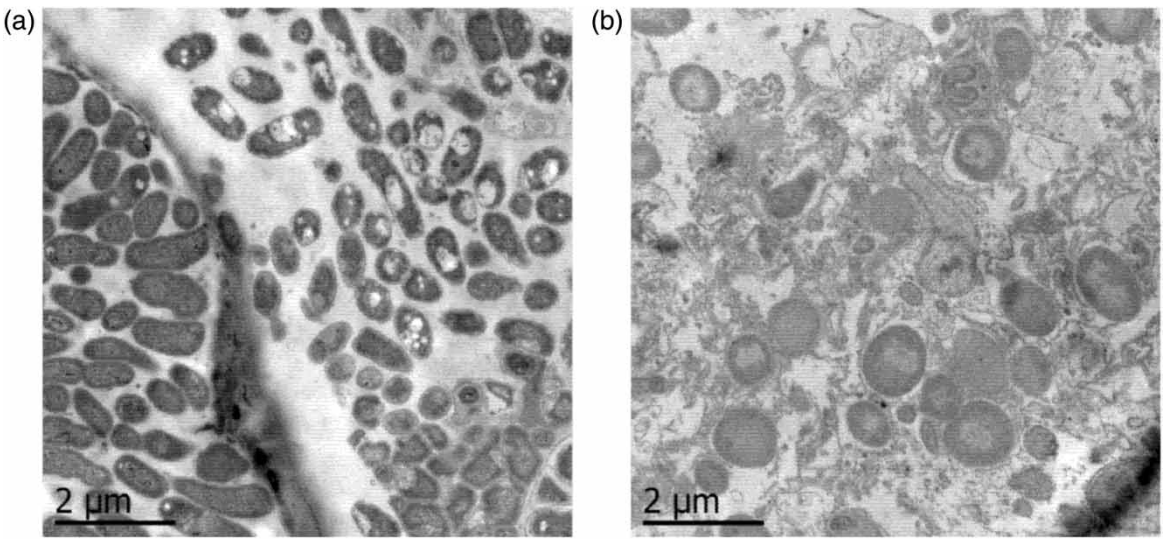

Figure 7 | TEM images at day 15. (a) Superficial fouling biofilm on membrane; (b) transversal cut showing the cells inside the membrane (non-reversible clogging).

the granules, playing an important role in the depletion of non-attached bacteria.

Chen & Lee (2011) have proposed that irreversible fouling is mainly caused by the soluble fraction of EPS, which is released to the liquid phase by various mechanisms. First the EPS, which bind microorganisms, are hydrolyzed into smaller pieces which are solubilized. Some metabolic products contribute also to this process (Meng *et al.* 2009). This material is then subsequently adsorbed on to the membrane, augmenting its thickness, and leading to an increment of the bacterial adhesion to the membrane surface, increasing the difficulty in its removal by the turbulence and backwashing.

Figure 7(a) shows the TEM analysis of the membrane surface on day 15. It is possible to observe the microorganisms forming a biofilm on the membrane. Micrographs suggest that on the membrane surface the cells form an exopolymeric matrix, with the EPS filling the space between cells (Figure 7(a)). Figure 7(b) shows a transversal cut of the membrane module, showing the penetration of some cells into the membrane and some EPS. This material is formed by polysaccharides and proteins, as discussed previously and agreeing with the results observed for the CMLS images. Also, the decrease in flux (from 12.5 to 7.8 L/(m^2·h)) and increase of TMP (15.4 to 50.2 kPa) observed after 15 days of operation of the reactor can be related to the accumulation of EPS. As a consequence of this non-reversible clogging, the membrane needed more than 10 h to permeation of the treated water.

Membrane biofouling mechanism in the aerobic granular reactor

Several factors contribute to the adsorption of polysaccharides to a membrane at the beginning of the biofouling, including membrane hydrophilicity (Li *et al.* 2008) and the exclusion size due to the structure of the polysaccharides (long and thin). The polysaccharides can be retained, thus increasing the blockage of the membrane pores (Shen *et al.* 2010). For an activated sludge MBR, a greater retention of carbohydrates (58–100%) compared with the protein (20–70%) has been reported despite the carbohydrates not being the main supernatant component (Drews *et al.* 2007; Dvorák *et al.* 2011). It also has been reported that EPS increase the liquid viscosity (Chu & Li 2005) resulting in a decrease of the filtration rate. The increase of polysaccharides accumulation on a membrane surface has been also related to an increment on cell lysis due to lack of substrate (Chen & Lee 2011).

Using the microscopy analysis and operational parameters, the membrane fouling can be explained by two mechanisms: (i) the mechanical inclusion of EPS and lysis material; and (ii) the development of a biofilm. According to Lee *et al.* (2008), a cake layer is the accumulation and compression of biological material rejected by the membrane and deposited on the membrane surface, whereas a biofilm is a community of microorganisms associated on a surface and encapsulated in an extracellular matrix. In the MSBR the fouling seems to be produced by the combination of both, a biofilm and a cake layer formation. In this sense, the term biolayer can be used to describe both phenomena. In a biolayer, the biofilm is the principal component and the cake layer, which is formed by flocs, EPS and metabolic products, participate to a lesser extent. With the results obtained, a biofouling mechanism on MSBR with granular sludge can be proposed considering the following five steps (Figure 8):

1. Adsorption of β-polysaccharides and their deposition in the inner pores of the membrane as well as on the surface, conditioning it for a subsequent colonization.
2. Deposition of free microorganisms and light flocs on the membrane surface (biolayer formation).
3. Growth of a biofilm by the multiplication of microorganisms to form a monolayer formed by a matrix of proteins with agglomeration and retention of microorganisms. At this point, some small granules and flocs are also retained at the membrane surface resulting in protuberances of the biological layer.
4. Clogging of the membrane by the deposit of cell debris, carbohydrates, microbial products.
5. Aging of the biolayer with eventual loss of some cells and debris.

Previous studies have described the biofilm formation. Flemming & Wingender (2010) explain that biofilm formation starts with the attachment of a cell to a surface. The role of protein and polysaccharides in biofilm formation is still an object of investigation (Berk *et al.* 2012). Our study is, to the best of our knowledge, the first approach to describe the fouling process in an MSBR with aerobic granules degrading an inhibitory compound, and with an emphasis on the polysaccharide and protein dynamics. The model proposed agrees to some extent with the previous results. We focused on the interactions between the granules and the membrane, showing that initial clogging of the membrane was started by polysaccharides, followed by a

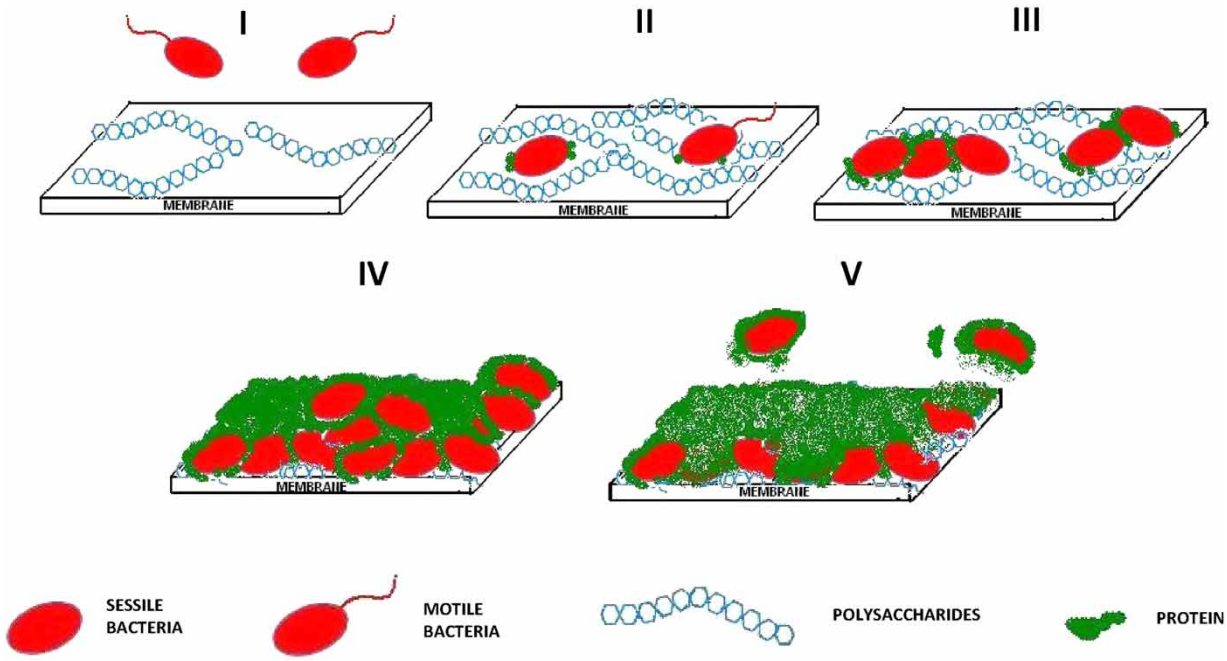

Figure 8 | A proposed mechanism for fouling biofilm.

bacterial colonization. In order to minimize the membrane fouling, it is necessary to avoid this bacterial colonization.

CONCLUSIONS

The fouling process of an aerobic membrane granular batch reactor degrading 4CP was described. It was observed that the main cause of the membrane fouling was the formation of a biolayer. The CMLS images revealed the stratification of EPS, and the accumulation of polysaccharides on the surface and inside the membrane. Proteins were mainly found on the surface of the membrane. The initial clogging of the membrane was started by polysaccharides followed by a bacterial colonization. The growth of the biofilm is due to the formation of a matrix of polysaccharides, proteins and an agglomeration of growing microorganisms.

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