An integrated approach to analyse biofilms of a full scale wastewater treatment plant

M. Martín-Cereceda, B. Pérez-Uz, S. Serrano and A. Guinea
Departamento de Microbiología III. Facultad de Ciencias Biológicas. Universidad Complutense de Madrid. Madrid 28040. Spain (E-mail: merm@ihm.ac.uk, cereceda@bio.ucm.es)

Abstract A rotating biological contactor (RBC) system operating in a full-scale wastewater treatment plant has been described by several approaches accounting for performance, composition and structure of biofilms in three stages through biological wastewater treatment (RBC1, RBC 2, RBC 3). RBC biofilms were effective in removing the BOD loading from 13 g BOD$_{5}$ d$^{-1}$ m$^{-2}$ in RBC 1 to 6 g BOD$_{5}$ d$^{-1}$ m$^{-2}$ in RBC 3. Analysis of biofilm composition showed: i) the volatile solids were similar in the three RBCs (0.6 g m$^{-2}$ VS per g m$^{-2}$ of TS); ii) the protozoan and metazoan biocenosis was mainly made up of ciliated protozoa, which were most abundant in RBC 2 (1.84 $\times$ 10$^6$ ciliates g$^{-1}$ VS). Relationship between ciliate species and physical-chemical profile of the system by cluster analysis indicated that the species Acineria uncinata, Amphileptus punctatus, Cinetochilum margaritaceum and Holosticha mancoidea were associated with the best RBC performance; iii) the exopolymeric matrix of the three RBC biofilms was mainly constituted by proteins, although humic substances, polysaccharides, uronic acids and DNA were also found. Analysis of biofilm structure by confocal microscopy indicated changes in biofilm organisation with depth. Results have been brought together and a graphic representation of the composition and architecture of RBC biofilms is presented.

Keywords Biofilm components; biofilm structure; ciliated protozoa; exopolymers; physico-chemical analysis; rotating biological contactors

Introduction
The rotating biological contactor (RBC) system is made of a series of closely spaced disks partially immersed in a tank through which wastewater flows. Its biological principle is based on the development of a thick and complex microbial film on the disk surface (Bishop and Kinner, 1986). The design of the RBC system in several compartments is believed to affect the biofilm composition since the physical-chemical and biological conditions are changed along the RBC train (Kinner and Curds, 1987).

Several studies focusing on specific aspects of the RBC system have been carried out (see Bishop and Kinner, 1986 for a review). The kinetics and RBC design (i.e. WEF/ASCE, 1991), the removal of nutrients and pollutants through the system (i.e. Qaisi and Qasem, 1996; Liu and Capdeville, 1996), the biofilm structure (Alleman et al., 1982; Kinner et al., 1983), and the microbial populations occurred during start-up and steady-state conditions (i.e. Madoni and Ghetti, 1981; Alleman et al., 1982; Kinner and Curds, 1987; Kinner et al., 1989; Chung and Strom, 1991; Luna-Pabello et al., 1996; Okabe et al., 1998; Pérez-Uz et al., 1998; Martín-Cereceda et al., 2001a,b) have been investigated. Nonetheless, RBC studies integrating several methods for biofilm analysis in a particular RBC system have not been so far addressed. Moreover, much of the RBC research has been done in laboratory or pilot-scale units, and these, being under constant and controlled conditions, do not exactly emulate the full-scale operation. Therefore, more studies at full-scale are necessary to better understanding the microbiology of the RBC and its relation with performance of this biological system.

The aim of this work is to make use of different approaches for an integrated descriptive characterisation of RBC biofilms. Inherent complexity of RBC biofilms is analysed in relation to their performance, composition and structure taking into account the
compartmentalisation as the distinctive feature of this system. Descriptive analyses must be a valuable support for subsequent insights on activity (function) and process control in wastewater treatment processes.

Material and methods

Sampling

Samples were collected from three units corresponding to first (RBC 1), intermediate (RBC 2) and final (RBC 3) stages in the biological wastewater treatment of a full-scale RBC system (surface area of 9,290 m² each unit; disk rotation speed of 1.6 rpm). RBCs are placed at a wastewater treatment plant (Northern Madrid, Spain) receiving urban inputs from a population of about 10,000 people. Water samples (1 litre) were taken of the circulating wastewater in the three RBC stages, and biofilm samples (23.75 cm²) were scraped using the top of a sampling bottle as a corer.

Determination of physical-chemical and biological variables

Physical-chemical profile. Five physical-chemical parameters (DO, BOD₅, NH₃-N, NO₂⁻-N, NO₃⁻-N) were measured in the RBC train wastewater (APHA et al., 1995) to monitor biofilm performance.

Biofilm protozoa and metazoa. Biofilm suspensions were obtained by dilution with 0.45 µm filtered raw wastewater to a final volume of 20 ml. Counts on these suspensions were made as described by Madoni (1988). Total solids (TS) and volatile solids (VS) were also measured on replicate samples of biofilm (APHA et al., 1995), and the number of protozoa and metazoa were referenced to grams of volatile solids. Ciliate identifications were conducted by silver carbonate and protargol methods (Wilbert, 1975; Fernández-Galiano, 1994). Several taxonomical guides were used (Kahl, 1930–35; Dragesco and Dragesco-Kernéis, 1986; Foissner et al., 1991–1995 and references therein).

Exopolymeric substances. Exopolymers were extracted by sonication (Jorand et al., 1995) and subsequent addition of a cation exchange resin (Dowex resin; Frølund et al., 1996). Exopolymers were recovered in the aqueous phase after centrifugation (30 minutes, 20,000g, 4°C). Colorimetric methods were employed to determine exopolymer chemical composition (Deriaz et al., 1949; Lowry et al., 1951; Dubois et al., 1956; Blumenkrantz and Asboe-Hansen, 1973).

Observation of biofilm architecture

Biofilm undisturbed strips were sampled of the RBC system and maintained in humid chambers to preserve hydration until microscopical observation. Biofilm architecture was analysed by Confocal Scanning Laser Microscopy (MRC1024, Bio-Rad, Hemel Hempstead, UK) in conjunction with several fluorescent dyes: DTAF (5-(4,6-dichloro-2-yl)aminofluorescein, Sigma-Aldrich Co.), PI (propidium iodide, Sigma-Aldrich Co.) and Congo Red (Sigma-Aldrich Co.).

Results and discussion

Biofilm performance

Biofilm activity during the monitoring period is shown in Figure 1. Mean values of organic loading dropped from 13 g BOD₅ d⁻¹ m⁻² in RBC 1 to 6 g BOD₅ d⁻¹ m⁻² in RBC 3, indicating that RBC biofilms acted efficiently in removing the organic matter from wastewater. Reduction of organic loading was related to a significant increase of dissolved oxygen (DO) concentrations along the RBCs (1.6 mg l⁻¹ in RBC 1 to 3.5 mg l⁻¹ in RBC 3).
nitrification took place from RBC 2 as revealed in the increase of nitrate loading averaged from 0.3 g NO₃-N d⁻¹ m⁻² to 0.5 g NO₃-N d⁻¹ m⁻² in RBC 3.

Biofilm composition

Total and volatile solids content. Total biofilm amount was highest in RBC 2 biofilms (155.4 g TS m⁻²). However, the Disk Biomass Index (DBI) was of 0.6 g of VS m⁻² g⁻¹ of TS m⁻² for all the stages. This indicates that the three RBC biofilms had the same organic matter per unit of dry weight with independence of thickness and external appearance of biofilm.

Protozoan and metazoan biocenosis. Observation and counting of protozoa (flagellates, amoebae, ciliates) and micrometazoa (rotifers, nematodes, annelids) by optical microscope showed a different spatial distribution of populations along RBC system (Figure 2). Amoebae (naked and testate) and micrometazoa were more abundant in RBC 3 biofilms, whilst heterotrophic flagellates were mainly on RBC 1 biofilms. The shift of protozoan and metazoa populations along steady-state RBC biofilms has been previously pointed out by authors (Bishop and Kinner, 1986). In pilot-scale biofilms, Hoag et al. (1980) also found that amoebae and metazoa were mainly present in the last RBC stages, and Kinner and Curds (1987) indicated that rotifers increased their number in the less loaded biofilms, which agrees with our results since in RBC 3 the organic load was reduced (see Figure 1).

Since the microbial biocenosis was primarily formed of ciliate protozoa, these were further studied. The highest ciliate mean abundance along the whole sampling period was found on RBC 2 biofilms (1.84 × 10⁶ ciliates g⁻¹ VS), while RBC 1 and RBC 3 showed similar densities (6.12 × 10⁵ ciliates g⁻¹ VS and 6.54 × 10⁵ ciliates g⁻¹ VS respectively). 55 ciliate species were identified in the three RBC stages, which was the higher number reported to date in RBCs (Hoag et al., 1980; Madoni and Ghetti, 1981; Kinner and Curds, 1987; Rivera et al., 1988; Kinner et al., 1989; Chung and Strom, 1991; Luna-Pabello et al., 1990, 1996).

The most abundant ciliates at each stage belonged to the peritrichous group, and were the following: Vorticella convallaria (60.4% of total abundance), Carchesium polypinum (14.4%) and Epistylys entzii (7.5%) for RBC 1; E. entzii (42.5%), V. convallaria (40.4%) and C. polypinum (12.5%) for RBC 2; V. convallaria (30.7%), E. entzii (16.9%), C. polypinum (12.8%) and Zoothamnium procerius (11.4%) for RBC 3. In Figure 3 is shown the spatial distribution of the most representative species of ciliates in the system. Changes in the type, abundance, and diversity of these species were observed along the RBCs, the RBC 3 biofilms holding the highest values of diversity and number of species. This is explained by the more propitious
environmental conditions of the RBC 3 stage: lower organic loading and higher oxygen content (Martín-Cereceda et al., 2001a, b for more details on RBC ciliate community).

In order to go inside the potential indicator value of ciliates in the RBC system, these species were related to physical-chemical RBC train parameters by statistical procedure. A numerical scale representing the mean values of physical-chemical parameters was assigned to each species, and species were then grouped by cluster analysis (Figure 4). Results show the existence of five assemblages of species in function of physical-chemical environment. A group formed by *Dexiotricha tranquilla*, *Litonotus crystallinus* and *Zoothamnium procerius* was associated with low BOD$_5$ values. Another group of species (*Acineria uncinata*, *Amphileptus punctatus*, *Cinetochilum margaritaceum*, *Holosticha mancoidea*, *Euplotes affinis*, *Oxytricha lanceolata*, *Drepanomonas revoluta*, *Spirostomum teres*, *Trochilia minuta* and *Glaucoma scintillans*) were related to high dissolved oxygen (DO) values. From this last group, the species *Acineria uncinata*, *Amphileptus punctatus*, *Cinetochilum margaritaceum* and *Holosticha mancoidea* were also linked to the lowest levels of BOD$_5$ and ammonia (NH$_3$-N), and the highest levels of nitrates (NO$_3$-N), being therefore associated with the best RBC performance. By contrast, two groups, one of them represented by *Acineta* sp.,

![Figure 2](https://iwaponline.com/wst/article-pdf/46/1-2/199/476870/199.pdf)

**Figure 2** Variation of the abundance of protozoan and metazoan groups along the RBC system. y-axis represents number of organisms (N) per g of volatile solids (g VS), and x-axis represents the whole sampling period at each of the three RBC units (RBC 1, RBC 2, RBC 3). GF: Green flagellates; HF: Heterotrophic flagellates

- *Acineta uncinata*
- *Amphileptus punctatus*
- *Cinetochilum margaritaceum*
- *Holosticha mancoidea*
- *Euplotes affinis*
- *Oxytricha lanceolata*
- *Drepanomonas revoluta*
- *Spirostomum teres*
- *Trochilia minuta*
- *Glaucoma scintillans*

![Figure 3](https://iwaponline.com/wst/article-pdf/46/1-2/199/476870/199.pdf)

**Figure 3** Spatial distribution (based on presence/absence) of the most representative species of ciliates in the RBC system
Paramecium aurelia and Tetrahymena pyriformis, and the other by Amphileptus pleurosigma, Opercularia articulata, Metopus es and Paramecium caudatum, indicated low levels of DO and high levels of BOD₅. Finally, a large group made up of 11 species was associated with intermediate values of physical-chemical parameters. This group included species very representative in many wastewater treatment plants, for example Aspidisca cicada, Vorticella convallaria, Litonotus lamella and Carchesium polypinum (i.e. Curds and Cockburn 1970a, b; Madoni, 1988; Madoni et al., 1993; Luna-Pabello et al., 1990, 1996).

Exopolymeric substances. Proteins, exopolysaccharides, humic substances, uronic acids and DNA were constituents of the EPS matrix, the proteins being the most abundant in all the RBC biofilms. This agrees with previous results in other types of biofilms (Table 1), in which sonication and/or Dowex resin have also been used for exopolymer extraction. Although the exopolymeric matrix accounted for around the same proportion in all the biological systems (29% of VS in RBC 1, 26% of VS in RBC 2 and 29% in RBC 3), a different quantity of each exopolymer was found in the three RBCs (Martín-Cereceda et al., 2001c).

Biofilm structure
Study of biofilm structure was approached by confocal microscopy (SCLM) because this technique has been reported very useful for the non-destructive examination of microbial aggregates. Results indicate similar biofilm architecture on all the RBC stages; however, a difference in the biofilm organisation with depth was observed. Outer biofilm layers had a higher variety of microorganisms with a clumped distribution in a highly porous exopolymeric matrix (Figure 5 a–f), while inner biofilm layers presented low microbial colonisation and a more packed organisation (Figure 5 g–h). Further details on confocal examination of RBC biofilms have been reported in Martín-Cereceda et al. (2001d).

All results were integrated together and a graphic representation of the composition and structure of RBC biofilms was depicted (Figure 6). RBC biofilms are complex biological aggregates colonised by a large variety of microorganisms: bacteria (individual and filamentous mats), protozoa (principally ciliates and naked and testate amoebae), green algae coverage and metazoa (rotifers, nematodes and annelids). This biocenosis was differentially disposed with depth, and ciliate protozoa were mostly found in the outer biofilm.
layers. Mineral particles and organic debris also appeared, and together with microorganisms, were embedded in a three-dimensional exopolymeric network of absorbed matter and microbial excreted substances, which is very important in biofilm dynamics.

Conclusions
In this study different methods for characterising the biofilms of a full-scale rotating biological contactor (RBC) system are presented. The main conclusions are:

1. The use of volatile solids content as a biofilm descriptor does not reflect the effect of RBC train compartmentalisation, providing very limited information for a comparative quantitative analysis of RBC performance.
2. Monitoring of the dynamics and composition of ciliated protozoa offers valuable information on the biological performance of the RBC system.
3. The composition of the RBC exopolymeric matrix is affected by the system compartmentalisation. This corroborates its dynamic nature and ability to respond to changes in the physical-chemical conditions and/or physiological state of colonising microorganisms.
4. Confocal microscopy is proved to be an effective tool for analysing in vivo the microbial heterogeneity and architecture of these mature biofilms.

Table 1  Literature reporting sonication and/or Dowex resin as methods to extract exopolymeric substances in biofilms. PolyS: Polysaccharides; ULS: Uronic like substances; HLS: Humic like substances

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<tr>
<th>Extraction method</th>
<th>Prots</th>
<th>PolyS</th>
<th>ULS</th>
<th>HLS</th>
<th>DNA</th>
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<tr>
<td>Dowex resin</td>
<td>351 mg g⁻¹ TOC</td>
<td>46</td>
<td>11</td>
<td>221</td>
<td>26</td>
<td>Jahn and Nielsen (1995)</td>
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<td>Dowex resin (centrif 12,000 g; 15 min.)</td>
<td>76 mg g⁻¹ TS</td>
<td>19</td>
<td>1.5</td>
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<td>Späth et al. (1998)</td>
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<tr>
<td>Dowex resin (2 h; 400 rpm)</td>
<td>200 mg g⁻¹ TS</td>
<td>25</td>
<td></td>
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<td>Azeredo et al. (1999)</td>
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<td>(centrif 9,000 g; 20 min.)</td>
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<td>Sonication (37 w; 1 min)</td>
<td>240 mg g⁻¹ TS</td>
<td>150</td>
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<td>Azeredo et al. (1999)</td>
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<td>(centrif 9,000 g; 20 min.)</td>
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<tr>
<td>Sonication (37; 1 min)</td>
<td>Dowex resin</td>
<td>142–157 mg g⁻¹ TS</td>
<td>31–48</td>
<td>1.6–3.4</td>
<td>19–44</td>
<td>15–25</td>
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<td>(100 g; 1 h; 400 rpm)</td>
<td>(centrif 20,000 g; 30 min.)</td>
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Figure 5  Confocal sections (x, y) of RBC biofilms; 5 a–e: Outer biofilm layers; a) Clumped distribution of bioaggregates. Arrow points at nematode; b–d: Filamentous bacteria mats, stalked peritrichous ciliates (arrow) and annelids (arrow head); e–f: EPS matrix (Congo Red staining) showing a network of channels that create a characteristic porous structure. 5 g–h: Inner biofilm layers. Observe the more homogenous biofilm organisation. White points in section g represent bacteria. Bars = 50 µm in sections b–d and g–h; 100 µm in sections e–f; 700 µm in section a
Acknowledgements

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References


Figure 6 Schematic representation of RBC biofilm architecture


