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# Confocal and Light Microscope Examination of Protozoa and Other Microorganisms in the Biofilms from a Rotating Biological Contactor Wastewater Treatment Plant

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**Summary.** Wastewater biofilms from a full-scale treatment plant by rotating biological contactor (RBC) system are described using confocal scanning laser microscopy (CSLM) and light microscopy (LM). The main objective was to determine the internal architecture of biofilms, with a particular emphasis on the spatial distribution of protozoa and their relation to other microorganisms and non-cellular components. Biofilms were composed of a wide variety of microorganisms: i.e. filamentous bacteria, green algae, ciliated protozoa, nematodes and rotifers. Peritrich ciliates constituted the most abundant group within protozoan and metazoan biofilm communities, and they played an important role in maintaining the multilayer biofilm organisation owing to the intricate entanglement formed by their peduncles together with the filamentous bacteria. Identification of ciliates to species level was approached by light microscopy, *Vorticella convallaria, Epistylis entzii, Carchesium polypinum, Vorticella infusionum* and *Opercularia articulata* being the most abundant species. It was shown by confocal microscopy that microbial distribution changed with biofilm depth, outer layers having the highest heterogeneity. Architectural biofilm organisation consisted of bioaggregates surrounded by an extensive exopolymeric matrix whose porosity decreased from outer to inner layers. The role of exopolymers as a structural component of RBC biofilms is also revealed by confocal microscopy. This study demonstrates the utility of confocal microscopy for the analysis of protozoan distribution within thick living wastewater biofilms.

**Key words:** biofilm architecture, ciliates, confocal scanning laser microscopy, full-scale wastewater treatment plant, metazoa, protozoa, rotating biological contactors.

## INTRODUCTION

Rotating biological contactor (RBC) system is one of the biological methods used for the sewage treatment. This system consists of a series of closely spaced discs partially immersed in a tank through which wastewater flows. Its biological principle is based on the metabolic activities of complex microbial communities that grow on the disc surface forming a biofilm (Bishop and Kinner 1986). Protozoa play a key role within biofilm microbiota; they are responsible of decreasing bacterial numbers contained in wastewater, and some of them have the capability to consume dissolved and flocculated organic matter (Bishop and Kinner 1986). Moreover, ciliates have been showed as bioindicators of biological process

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efficiency (Kinner and Curds 1987, Kinner *et al.* 1989, Luna-Pabello *et al.* 1990).

The spatial distribution of these microorganisms and their interactions within biofilm structure has been scarcely studied. To date, architecture of RBC biofilms has been only addressed by scanning and transmission electron microscopy (Alleman *et al.* 1982, Kinner *et al.* 1983); however, as these techniques require a previous sample dehydration and fixation, they do not allow for a non-disturbing architectural observation. Confocal scanning laser microscopy (CSLM), due to its optical sectioning features, high spatial resolution and minimal sample manipulation, is a very powerful tool for the non-destructive examination of thick biological samples (Caldwell *et al.* 1992, Laurent *et al.* 1994). Recently, confocal microscopy has been successfully applied to different aspects of microbial ecology. Bacterial dynamics in soil smears (Bloem *et al.* 1995), structure of suspended flocs from freshwater (Droppo *et al.* 1996, 1997; Liss *et al.* 1996; Neu 2000) and from activated sludge wastewater treatment system (Wagner *et al.* 1994a, b; Droppo *et al.* 1996; Liss *et al.* 1996; Wagner *et al.* 1996; Olofsson *et al.* 1998), have been described using CSLM. Spatial arrangement, composition and properties of different types of microbial biofilms have also been approached by confocal microscopy: studies of bacterial monoculture biofilms (Korber *et al.* 1994, Kuehn *et al.* 1998), laboratory-grown mixed biofilms (Lawrence *et al.* 1991, Stoodley *et al.* 1994, Wolfaardt *et al.* 1994, Massol-Deyá *et al.* 1995, Hausner and Wuertz 1999), and natural biofilms colonising plant leaf surfaces (Morris *et al.* 1997), or reactors treating river waters (Neu and Lawrence 1997) and petroleum-contaminated groundwaters (Massol-Deyá *et al.* 1995), have revealed the heterogeneous biofilm organisation.

However, there are hitherto no studies on the application of CSLM to reveal the occurrence *in situ* of protozoa within mature biofilms of full-scale wastewater treatment plants. Therefore, the main aim of this work was to provide a first descriptive report on the spatial distribution of protozoa (and other) microorganisms within RBC biofilms using confocal microscopy. Arrangement of non-cellular components (i.e. exopolymers and debris) and their relation to biofilm microbial community was also outlined through this microscopical technique. Confocal microscopy approach was complemented with light microscopy (LM) to get further information concerning abundance and identification of protozoan species in the biofilms.

## MATERIALS AND METHODS

### Sampling

Biofilm samples (6 individual samples) were taken from the external discs of a four series RBC system (surface area 9290 $m^2$ each; organic loading 0.006-0.01 Kg $BOD_5$ $m^2$ $d^{-1}$) at a full-scale wastewater treatment facility ("Las Matas", North of Madrid, Spain) treating domestic sewage derived from a population of 10,000 people. Biofilm thickness ranged between 0.4-0.5 cm, but sometimes biofilms could reach up to 1 cm thick. Sampling of biofilm was done by pressing a spatula against the disc surface and scrapping carefully biofilm strips preserving biofilm architecture. These were placed on microscopical slides, kept in humid chambers to maintain hydration, and transported to the laboratory in a refrigerated container.

### Biofilm processing

Samples were maintained in humid chambers to preserve hydration during study and examined within 2-3 h of collection. Biofilm segments of 5 by 5 mm were carefully sliced avoiding disruption using a razor blade, and stained with DTAF (5-(4,b-dichlorotriazin-2-yl) aminofluorescein, Sigma-Aldrich Co.; 2 µg/ml PBS) and PI (propidium iodide, Sigma-Aldrich Co.; 10 µg/ml PBS). DTAF labels proteins with green fluorescence, while membrane-impermeable PI labels nucleic acids of damaged (dead) cells with red fluorescence. Thus, PI dye was used to assess cell viability. To detect the exopolysaccharide matrix, some biofilm samples were only stained with a sugar non-specific fluorescent marker (CR, Congo Red, Sigma-Aldrich Co.; 10 µg/ml PBS) (based on Allison and Sutherland, 1984). After 40 min at room temperature all stained samples were rinsed with PBS to remove excess of fluorochromes, deposited on coverslips and imaged by confocal microscope. Outer biofilm layers were those oriented to the liquid phase flow and inner biofilm layers were those in contact to the RBC support.

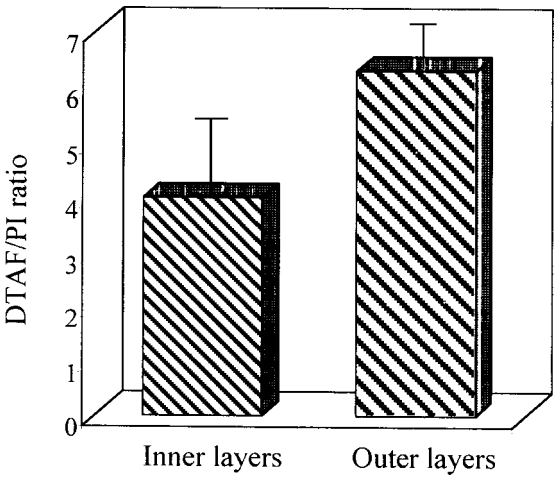
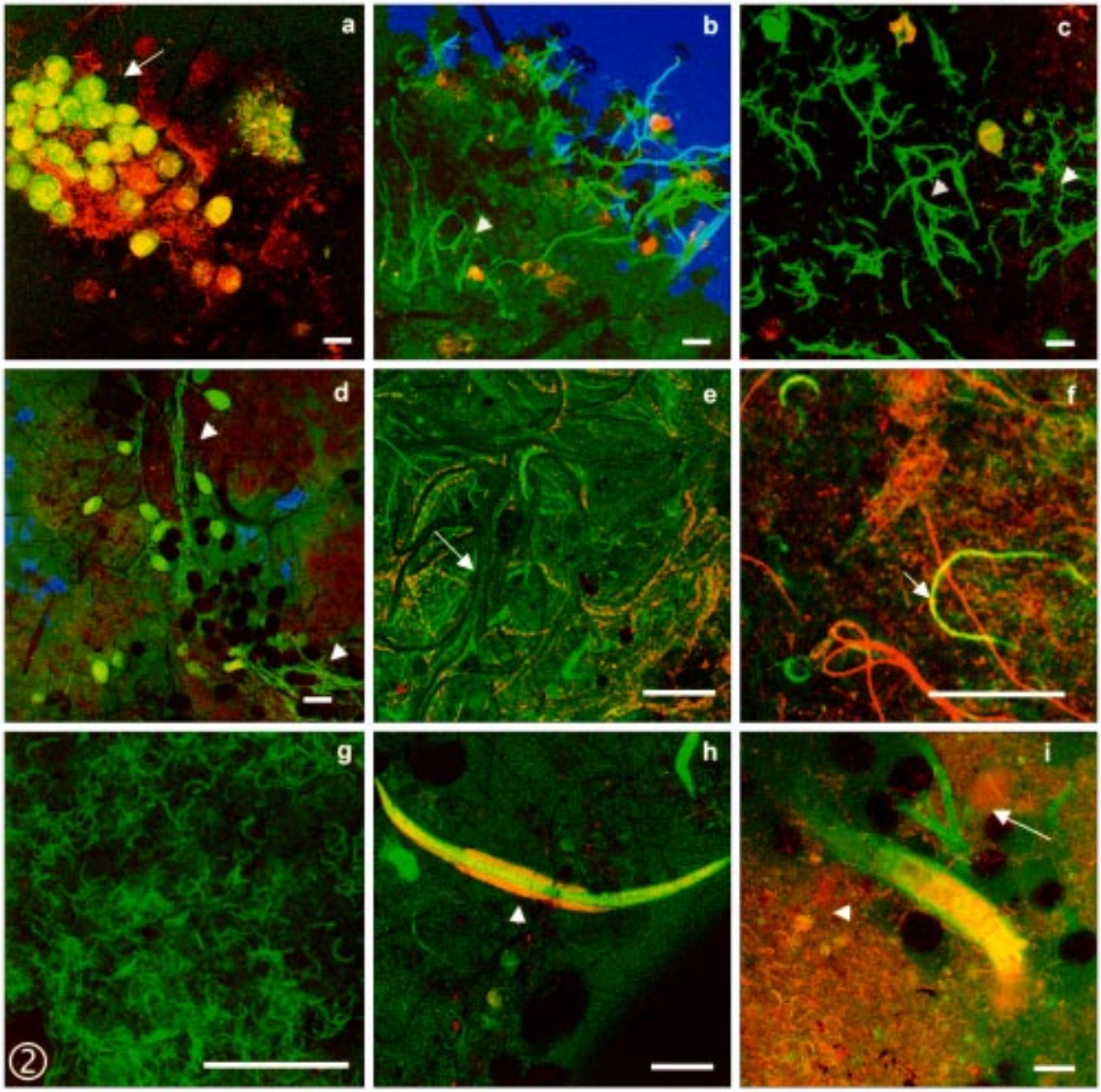



**Fig. 1.** DTAF/PI ratio (determined as the average DTAF value to the average PI value of images from each depth) in both inner and outer layers of the RBC biofilms studied




**Figs 2 a-i.** Single horizontal CSLM sections stained jointly with DTAF and PI dyes illustrating the microbial diversity in the outer RBC biofilm layers. **a**- peritrich ciliated protozoa colonies (arrow) like *Carchesium polypinum* were usually observed. **b-d** - entanglement of peritrich peduncles (arrow head) maintained multilayer biofilm arrangement. **e-f** - extensive filamentous bacterial mats (*Beggiatoa* spp.) provided consistence to the biofilm (arrow). **g** - green algae of genus *Selenastrum* occupied wide areas of the outer biofilm. **h-i** - aerobic nematodes (arrow head in h) were common inhabitants of these regions. Note the presence of peritrich ciliates (arrow in i) and *Selenastrum* cells (arrow head in i). Scale bar - 50 µm, except **b** and **d** - 100 µm. Colour coding: green - DTAF; red - Congo Red; blue - transmission signal (phase contrast)

## Confocal microscopy examination

A MRC-1024 confocal scanning laser microscope (Bio-Rad, Hemel Hempstead, UK) coupled to an inverted microscope (Eclipse TE300 Nikon) was used to analyse biofilm architecture. DTAF and PI were excited at 488 nm and fluorescence emission was collected through BP 515/15 nm and BP 605/20 nm respectively. Congo Red stained samples were examined at the same wavelength as set for PI. Observations were made with the following fluorite lenses (Nikon): x20 (0.45 Numerical Aperture, NA), x40 (0.60 NA) and x63 -oil immers-

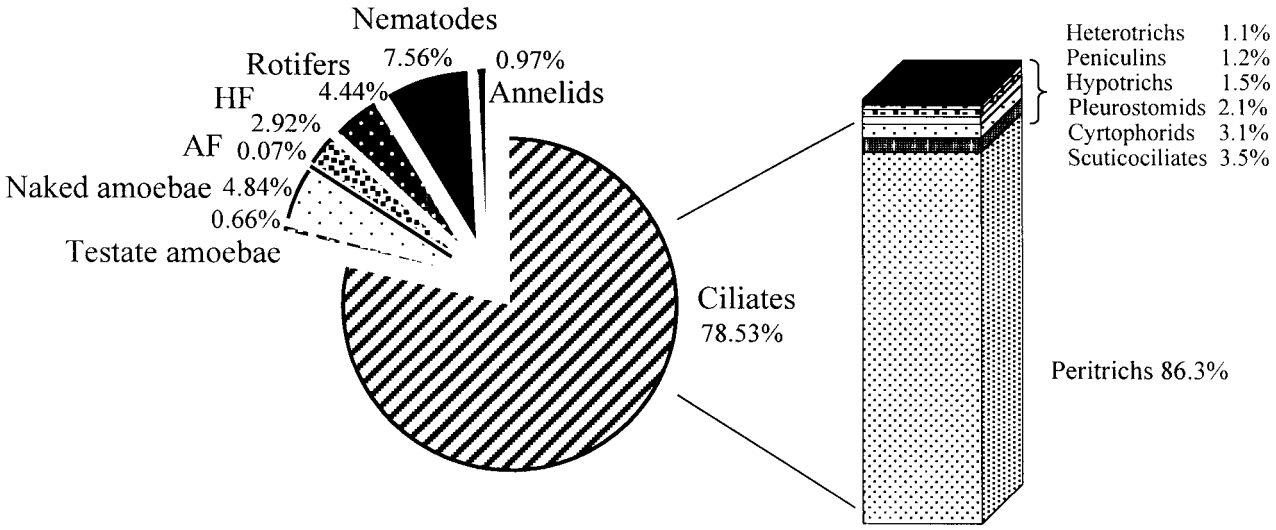



**Fig. 3.** Relative abundances (%) of protozoan and metazoan communities in the RBC biofilms studied, and distribution of ciliate abundances into taxonomic groups (Order). HF - heterotrophic flagellates; AF - autotrophic flagellates

ible (1.4 NA). Twenty five single images were recorded for each of the 6 individual samples collected. In some cases, sequences of images were taken along the optical Z axis using the confocal optical sectioning facility. Image acquisition was performed with the software package delivered with the instrument (Lasersharp v.3.2.). A minimum processing of samples was done with the program Confocal Assistant (v.4.02).

## Identification of microorganisms

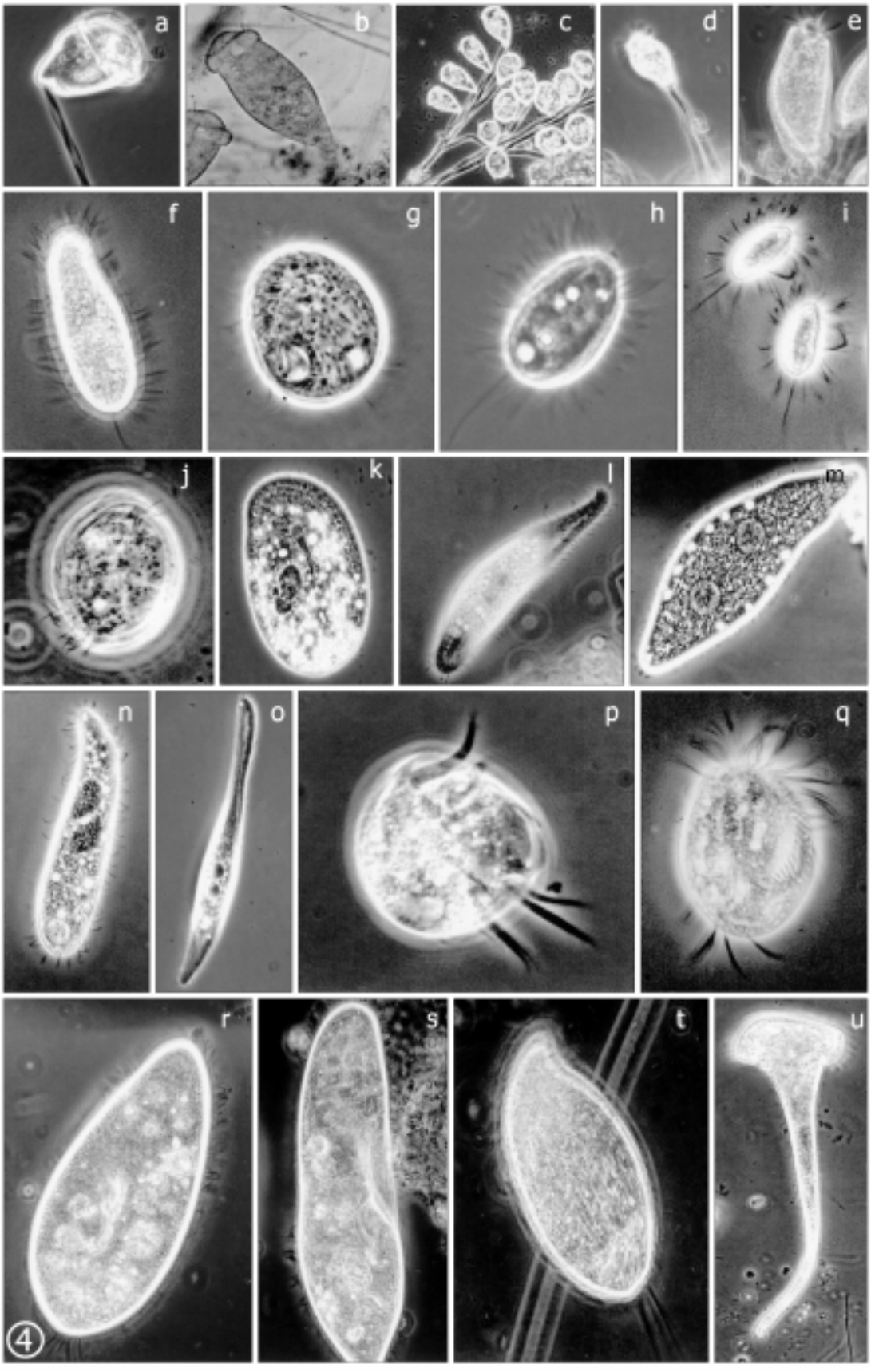
Replicate samples of biofilm were collected to determine abundances of protozoan and metazoan groups, and to conduct microorganism identifications. These samples were mixed with 0.45 µm filtered bulk wastewater liquor, and the biofilm suspensions obtained were then analysed through light microscopy (bright field and phase contrast). Counts were made as described Madoni (1988) for activated sludge system. To characterise ciliates to species level, pyridinated carbonate (Fernández-Galiano 1994) and protargol (Wilbert 1975) methods were applied. Several taxonomical works were used to help in the identifications (Kahl 1930-35; Foissner *et al.* 1991, 1992, 1994, 1995 and references therein). Specialised literature on metazoa and algae were also checked (Edmondson 1959, Pentecost 1984 and references therein).

## RESULTS AND DISCUSSION

Examination by confocal microscope was made in the outer layers (up to 200 µm from external surface) and the inner layers (up to 200 µm from the disc support) of the removed RBC biofilms. A different distribution of fluorescence intensity was observed with depth; DTAF/PI ratio was higher in outer layers (Fig. 1), owing to the higher DTAF signal and the lower PI signal obtained in these layers. These results indicate that microbial biomass and architecture of biofilm matrix changed with depth.

In outer layers, microbial biocenosis was very heterogeneous (Fig. 2). Complex communities mainly constituted by filamentous bacteria, protozoa, green eukaryotic algae and small metazoa were observed. Filamentous bacteria in the RBC plant studied (Figs 2 e, f), which were mainly represented by *Beggiatoa* spp. (Galván *et al.* 2000), were entangled and forming a wide coverage

**Figs 4 a-u.** Light microscope (phase contrast) microphotographs of the most representative species of ciliates in the RBC biofilms studied. **Peritrichs: a** - *Vorticella convallaria* (x270); **b** - *Epistylis entzii* (x180); **c** - *Carchesium polypinum* (x60); **d** - *Vorticella infusionum* (x225); **e** - *Opercularia articulata* (x255). **Scuticociliates: f** - *Dexiotricha tranquilla* (x640); **g** - *Cinetochilum margaritaceum* (x880); **h** - *Uronema nigricans* (x990); **i** - *Cyclidium glaucoma* (x530). **Cyrtophorids: j** - *Trochilia minuta* (x900); **k** - *Trithigmostoma cucullulus*(x245). **Pleurostomatids: l** - *Litonotus lamella* (x320); **m** - *Amphileptus pleurosigma* (x230); **n** - *Acineria incurvata* (x350); **o** - *Litonotus cygnus* (x230). **Hypotrichs: p** - *Aspidisca cicada* (x1000*)*; **q** - *Euplotes affinis* (x460). **Peniculins: r** - Paramecium aurelia complex (x400); **s** - *Paramecium caudatum* (x250). **Heterotrichs: t** - *Metopuses* (x430*)*; **u** - *Stentor roeselii* (x90)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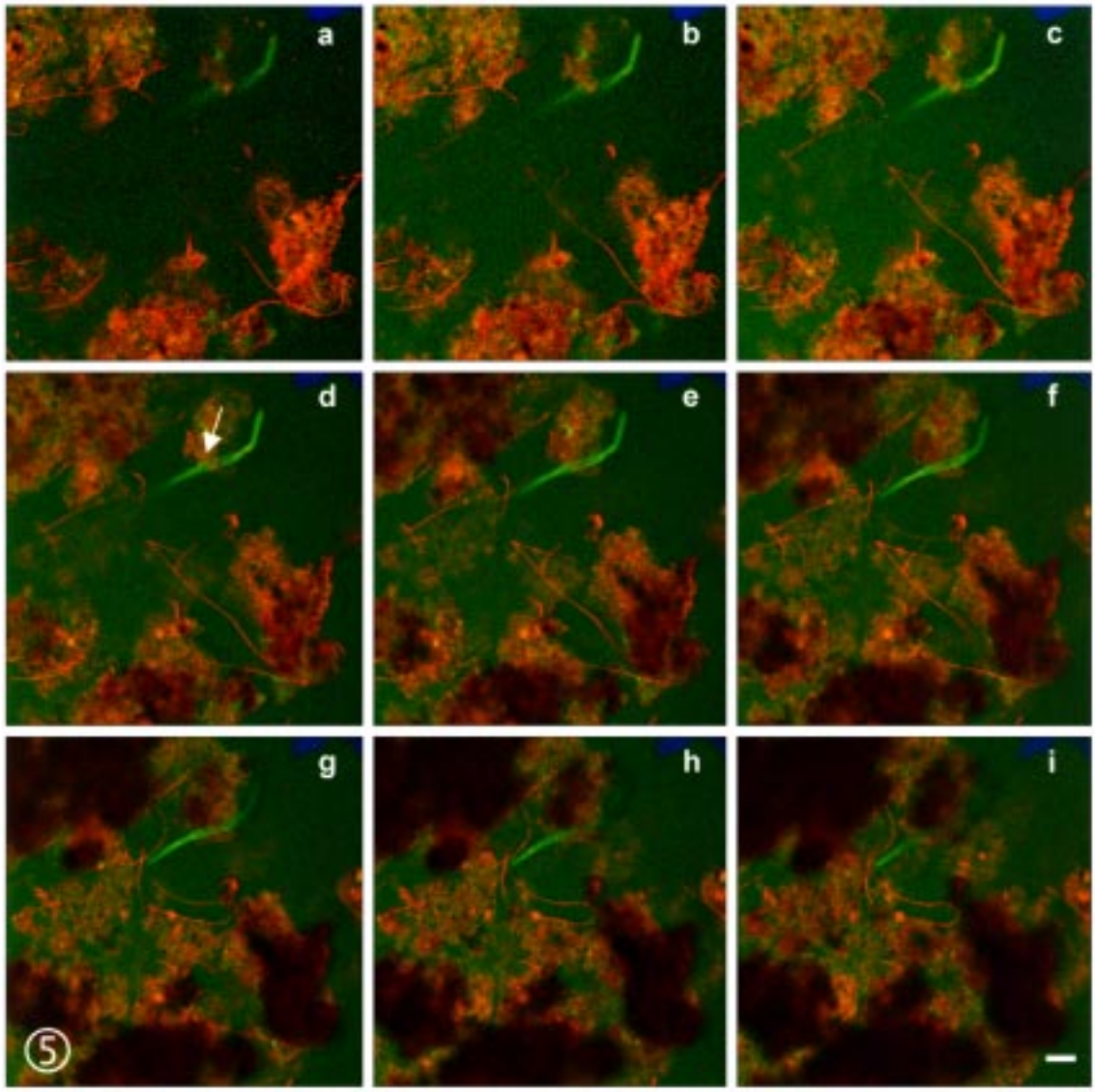
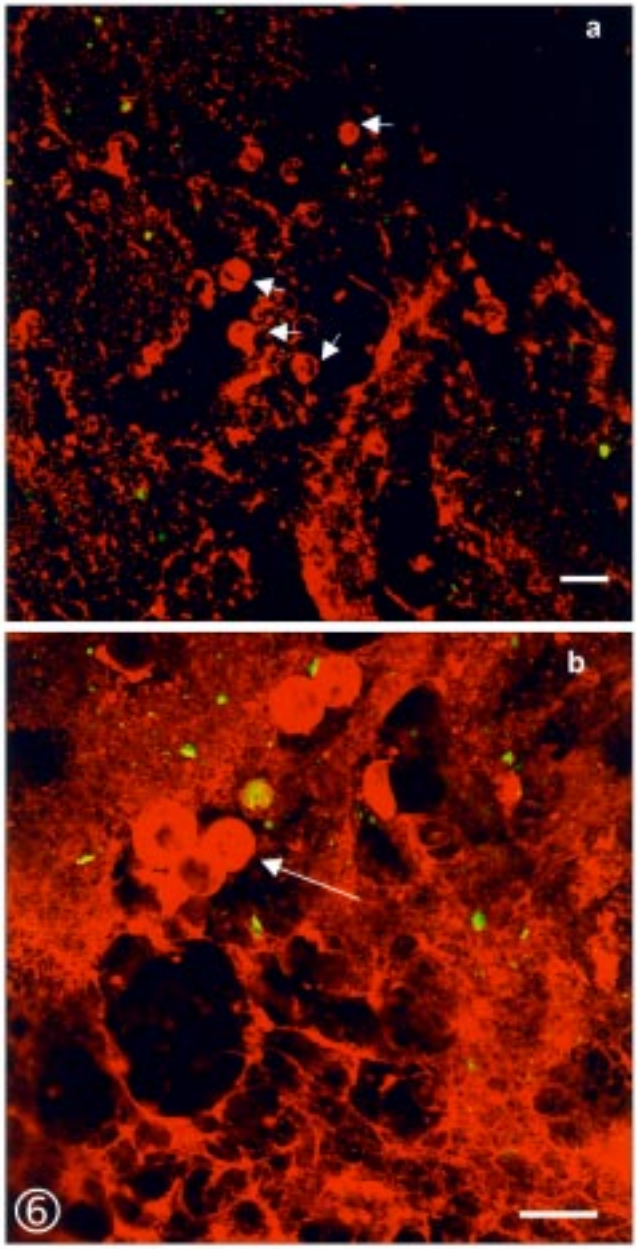



**Figs 5 a-i.** Series of horizontal CSLM sections of the outer RBC biofilm layers stained jointly with DTAF and PI dyes. Images are arranged towards the outermost surface of biofilm. Sections were taken at 10 µm intervals. Note the organisation in bioaggregates scattered on the proteic sheet (green fluorescence of DTAF), and the organic or inorganic material incorporated to biofilm surface (see g, h, i, sections). Arrow points a live nematode individual. Scale bar - 100 µm

within the biofilm. Confocal microscope observations denoted that peritrich ciliates were the most abundant group of protozoan and metazoan communities in these RBC biofilm layers. Peduncles of these ciliates appeared connected between and together with the filamentous bacteria, resulting in a complicate network that supports the multilayer biofilm organisation (Figs 2 a-d). Abundances of protozoan and metazoan communities in biofilm suspensions were obtained through light microscope counts, confirming that the peritrich ciliates hold around 90 % of total abundance (Fig. 3). Other ciliate groups (Order) represented in the biofilms were scuticociliates, cyrtophorids, pleurostomatids, hypotrichs, peniculins and heterotrichs, but their abundances were extremely lower than that of the peritrich group (Fig. 3). Representatives of tetrahymenids, suctorians gymnostomids, nassulids and prostomids were also occasionally found in RBC biofilms, and they accounted together for less than 1% of total ciliate abundance. Using light microscopy in conjunction with silver nitrate methods, the biofilm ciliate species were characterised to species level. The peritrich ciliates were mainly

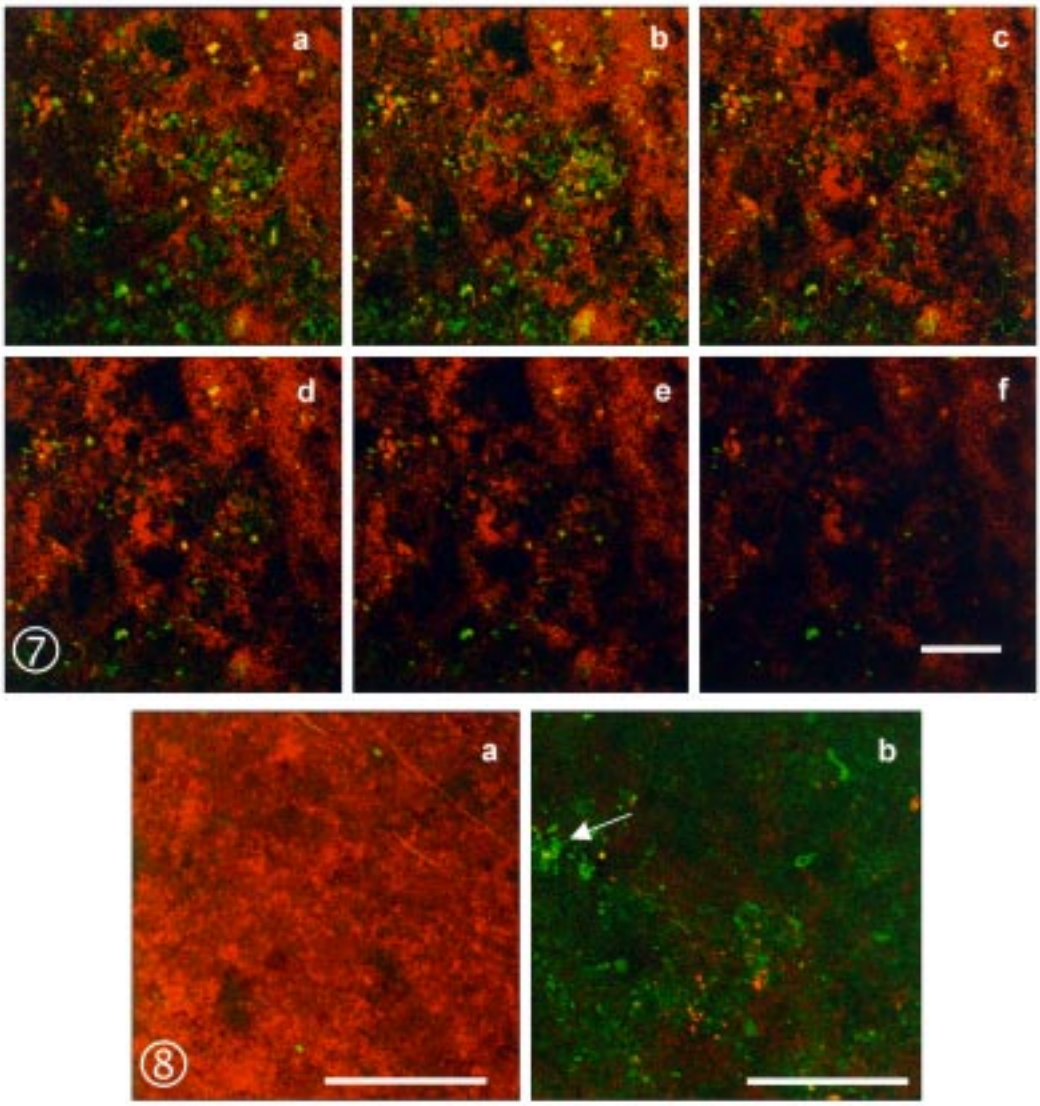



**Figs 6 a, b.** Exopolysaccharide matrix, stained with Congo Red dye and visualised by CSLM, in the outer RBC biofilm layers. Macropored organisation made of intersticies, voids and interconnecting fibrils. Spherical structures (arrow) are peritrich zooids having cell-wall mucopolysaccharides revealed by Congo Red staining. Scale bars - 100 μm

represented by *Vorticella convallaria, Epistylis entzii, Carchesium polypinum, Vorticella infusionum* and *Opercularia articulata* (Figs 4 a-e). Other representative species of ciliates in the RBC biofilms studied are shown in Fig. 4. These species were rarely seen when using confocal microscope, owing to their lower abundance and their fast movement.

The architecture of outer layers was depicted by a patchy distribution of microbial aggregates dispersed in a smooth organic layer with high protein content, as showed the green staining with DTAF (Fig. 5). The outermost regions had material not labelled with the fluorochromes used (see Figs 5 g-i - brownish areas in sections). This material seems to be suspended or colloidal organic debris and mineral particles, which provide additional surfaces for microbial colonisation rendering in a heterogeneous biofilm development (Neu and Lawrence 1997). A widespread network of carbohydrate extracellular material was revealed by Congo Red staining (Fig. 6). This network had a very porous organisation, with numerous interstices and channels interconnected by fibres that represent a backbone matrix binding together biofilm components. Zooids of peritrichs were usually surrounded by this carbohydrate component as it can be seen in Fig. 6. These results are consistent with those observed by Droppo *et al.* (1996) in activated sludge flocs, in which the exopolysaccharide matrix is described as a fibrous-textured net with sorption properties. Channels appear as stable components of biofilm architecture, and they could be due to the high presence of protozoa and metazoa in the RBC biofilms studied. These microorganisms, by their continuous movements and the predation on bacteria, may contribute to restore the voids and maintain the porosity (Massol-Deyá *et al.* 1995). According to some authors (de Beer *et al.* 1994, Lawrence *et al.* 1994), the formation of channels in thick biofilms seems to be a microbial strategy to solve the problem of nutrient and gases transport towards inner layers.

Inner layers of RBC biofilms had a more uniform and compact aspect. DTAF staining of bacteria was reduced in the most internal zones (Figs 7 d-f sections), revealing the decrease of bacterial biomass with depth. This vertical stratification of biomass in wastewater biofilms agrees with that previously reported in other biofilms, since using microslicing techniques (Okabe *et al.* 1996, Zhang and Bishop 1997) or CSLM (Massol-Deyá *et al.* 1995, Neu and Lawrence 1997), it was also revealed that microbial density was reduced in the innermost biofilm layers. Generally, inner layers had a larger percentage of non-viable bacteria than the outer layers, as it was revealed by their higher PI signal (Fig. 8 a), and the lower proportion of protozoa and metazoa. Peritrich ciliates were very rarely seen in these layers, and concerning metazoa, only nematodes were sporadically present. This fact is explained because the majority of protozoa and metazoa identified in these




**Figs 7, 8.** Spatial organisation of the inner RBC biofilm layers. Biofilms were stained with both DTAF and PI dyes. **7** - series of horizontal CSLM sections arranged towards the innermost layers. Sections were taken at 10 µm intervals. Live biomass (green fluorescent staining) appeared to be decreased in the deepest layers (last sections). Scale bar 50 µm. **8 a** - putative dead cells stained with PI (red fluorescence) were viewed in higher proportion in inner layers. **8 b** - putative live bacterial clusters (arrow) stained with DTAF (green fluorescence) were also noticed in inner layers. Scale bars - 50 µm

RBC biofilms hold an aerobic metabolism (Martín-Cereceda *et al.* 2001a, b), and oxygen availability is recognised to be very low in the deep layers of wastewater biofilms (Bishop and Kinner 1986). In this sense, only the anaerobic ciliate M*etopus es* was at times observed in the inner biofilm layers. Presence of viable bacteria clumps in the internal zones (Fig. 8 b) was observed, and these could be involved in the attachment of the basal layer to the disc support (Massol-Deyá *et al.* 1995).

Polysaccharide exopolymeric matrix (Congo Red staining) of inner layers presented low porosity. The less macro-pored organisation is probably due to the higher biofilm compacting with age, the inner being the oldest layers (Bishop *et al.* 1995). Exopolysaccharide inner material is characterised by enlarged and amorphous structures, and by a laminate matrix with diffuse appearance addressed towards outer layers (Fig. 9). Presence of extracellular polymeric material proposes that at least a part of the biomass existing in inner layers is active (Eighmy *et al.* 1983).

Extracellular polymeric substances (EPS) are an important component in biological aggregates since they create a highly hydrated 3-D network which embraces

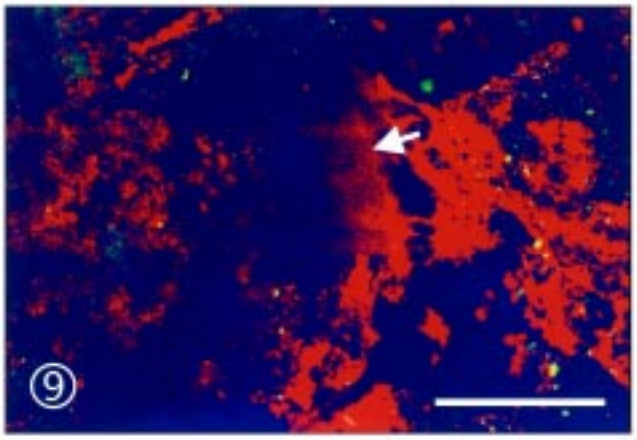



**Fig. 9.** Exopolysaccharide matrix, stained with Congo Red dye and visualised by CSLM, in the inner RBC biofilm layers. Note the mucoid-like appearance of matrix (arrow head). Scale bar - 100 μm

together microorganisms, organic and inorganic particles (Characklis and Marshall 1990, Neu 1994). Exopolymers represent approximately 28% of volatile solids content (VS) in the RBC biofilms studied, proteins (17% of VS) and polysaccharides (4% of VS) being the most abundant components (Martín-Cereceda *et al.* 2001c). Microbial excretion is believed one of the main origins of extracellular substances, so the conspicuous presence of exopolymeric materials in the RBC biofilms studied seems to be an indicator of active metabolism inside these biofilms. Exopolymers would be involved in different aspects of biofilm dynamic (Lappin-Scott *et al.* 1992, Neu 1994, Neu and Lawrence 1997): (i) representing a structural component; (ii) permitting attachment to the disc; (iii) supplying an extensive surface area for colonisation of bacteria, protozoa and metazoa; (iv) contributing to assemble the bioaggregates serving as a glue among microorganisms, and (v) providing a site for the sorption of soluble nutrients and suspended or colloidal material to be integrated inside biofilm structure.

The high nutrient content and the variety of products coming in raw sewage determine a large structural complexity and developing of wastewater mature biofilms. Adequate techniques are needed to the undamaged revealing of biofilm organisation. This study has demonstrated that confocal microscopy is perfectly suited for the easy, direct, and non-destructive analysis of such a thick biofilm. Our results indicate that microbial communities of wastewater biofilms grow up along discrete aggregates surrounded by an exopolymeric matrix rich in interstitial voids. These results are consistent with the modern concept of biofilm architecture describing biofilms as cluster of cells embedded in a complex EPS matrix with abundant water-permeable channels (Lawrence *et al.* 1991, Caldwell *et al.* 1992, Massol-Deyá *et al.* 1995), rather than with the traditional view of a homogeneous biofilm (Lock *et al.* 1984).

In this study, confocal microscopy has been applied for the first time to obtain undisrupted images of the protozoan distribution within wastewater biofilms, revealing how is the microenvironment around the protozoa and the *in situ* interactions with other microbial and non-cellular components. It is suggested that protozoa play a fundamental role in the structure of RBC biofilms since they are involved in the spatial biofilm organisation by maintaining the stability of multilayer arrangement and by contributing to the biofilm porosity.

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