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Involvement of Crawling and Attached Ciliates in the Aggregation of Particles in Wastewater Treatment Plants

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Abstract: The biological community in activated sludge wastewater plants is organized within this ecosystem as bioaggregates or flocs, in which the biotic component is embedded in a complex matrix comprised of extracellular polymeric substances mainly of microbial origin. The aim of this work is to study the role of different floc-associated ciliates commonly reported in wastewater treatment plants-crawling *Euplotes* and sessile *Vorticella*- in the formation of aggregates. Flocs, in experiments with ciliates and latex beads, showed more compactation and cohesion among particles than those in the absence of ciliates. Ciliates have been shown to contribute to floc formation through different mechanisms such as the active secretion of polymeric substances (extrusomes), their biological activities (movement and feeding strategies), or the cysts formation capacity of some species. Staining with lectins coupled to fluorescein showed that carbohydrate of the matrix contained glucose, manose, N-acetyl-glucosamine and galactose. Protein fraction revealed over the latex beads surfaces could probably be of bacterial origin, but nucleic acids represented an important fraction of the extracellular polymeric substances of ciliate origin.

Keywords: ciliates, wastewater treatment, bioaggregation, extracellular polymeric substances (EPS)

Introduction

Ciliates are one of the most important populations of the biological communities in the aeration tanks of wastewater treatment plants (WWTP) with activated sludge systems. These microorganisms are essential for the optimal performance of sewage treatment being involved in the removal of bacterial populations through grazing, including pathogenic species (Curds and Fey, 1969). As they are very sensitive to environmental conditions, ciliated protozoa dynamics and community structure have been widely used as indicators of the operating conditions of WWTP (Curds, 1975; Madoni et al. 1993, 1996; Madoni, 1994; Salvadó et al. 1995, Martín-Cereceda et al. 1996). Besides, several authors have pointed out their probable contribution to development of aggregates or flocs by secretion of polymeric compounds (Watson, 1945; Curds, 1963; Arregui et al. 2007).

Activated sludge flocs are aggregates of organic and inorganic particles, plus a microbial community and extracellular microbial polymers, mostly carbohydrates. These substances form a complex network surrounding dead and living cells that facilitate their sedimentation in what it is denominated sludge and therefore this promotes clarification of the effluent. Flocs vary in size from less than 10 μm up to 1mm (Jenkins et al. 2003).

Depending on their ecological niche inside the reactor, ciliates can be divided into two categories: species associated to flocs -crawling and sessile ciliates- and swimming ciliates that move freely in the mixed liquor. Crawling ciliates are adapted to move on the floc and they usually show a flattened body with cilia mainly located just on one cell surface, which are frequently organized in specialized associations (i.e. cirri of spirotrichs). Sessile ciliates are permanently associated to flocs due to the presence of attachment structures such as stalks or mucous loricas (Madoni, 1994).

The aim of this work is to demonstrate the active role of ciliates in the formation of particle aggregates or flocs. For this purpose, we have employed three representative species of ciliates associated to flocs: a crawling ciliate of the genus *Euplotes* and two species of the genus *Vorticella*, typical sessile ciliates reported in wastewater treatment plants.

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Material and Methods

Microorganisms and culture conditions

Euplotes sp. was isolated in our laboratory from samples collected from the aeration tank of an activated sludge sewage plant (Madrid, Spain). Direct isolation was done with a Pasteur micropipette using a stereomicroscope. Cultures were maintained in Chalkley medium adding periodically a suspension of *Escherichia coli* as food.

Vorticella similis (CCAP 1690/2) and *Vorticella microstoma* (CCAP 1690/3) were also maintained in Chalkey medium supplied with *E. coli* as food.

Cultures of *Euplotes* sp. and *Vorticella* spp. were incubated at 18 ºC in the dark.

Exocytosis experiments

Alcian Blue (Sigma, CAS 75881-23-1) was used as inductor of cell exocytosis, following the protocol described in Turkewitz et al. (1999).

Aggregation experiments

Aggregation of latex beads was followed in a set of experiments with ciliates. An aqueous suspension of latex beads (Sigma Polystyrene Latex Beads SD 26-diameter 21.1 μm) was added to ciliate cultures with bacteria (1 μl:500 μl). Two control experiments were performed at the same time: (i) latex beads incubated in the sterile culture medium and (ii) latex beads in the culture medium and the same volume of bacterial suspension that was added to the ciliate culture.

Progress of experiments was followed under a ZEISS STEMI S V6 or a NIKON SMZ-2T stereoscopic microscope.

Determination of extracellular polymeric substances (EPS) composition

EPS composition was tested for carbohydrates, proteins and DNA. Characterization of these exopolymeric matrix components of the flocs was carried out using different fluorochromes. Three different lectins were assayed to stain carbohydrates: Con A (from *Canavalia ensiformis*, Sigma C7642) reacting specifically with D-manose and D- glucose, PNA (from *Arachis hipogaea*, Sigma L7381) with D-galactose, and WGA (from *Triticum vulgare*, Sigma L4895) which reveals N-acetyl-D-glucosamine residues. All of them were coupled to fluorescein. Working and stocking solutions were prepared following Wilks and Sleigh (2004) procedures.

Samples were incubated with latex beads (1:500 μl) and each lectin (50 μg/ml) for 20 min and then washed three times with phosphate buffer saline (PBS). Lack of unspecific binding to the latex beads was tested performing controls with the specific carbohydrates. Lectins were also assayed on ciliate cultures (200 cells/500 μl) without latex beads.

Aliquots of experiments were also treated with DTAF (Sigma D0531) for revealing proteins, and propidium iodide (Sigma 81845) to detect DNA of non-viable cells. Double staining was performed incubating samples for 40 min in the presence of both fluorochromes diluted in PBS to a final concentration of 2 μg/ml and 10 μg/ml respectively. The excess of staining was washed off with PBS.

Microscopy

Preparations were observed using a Zeiss Axioplan epifluorescence microscope. Images were acquired with a CCD spot Camera and treated with the software MetaMorph Imaging System (Universal Imaging System).

Results

Exocytosis experiments

Experiments with alcian blue showed that *Euplotes* sp. secreted a polymeric material, blue dyed, that remained close to the ciliate surface in most individuals. A patent stain of two rounded structures was observed in the cytoplasm, close to the oral cavity (Fig. 1).

The induced exocitosis in *V. similis*, showed blue dyed rounded structures distributed densely packed within zooids cytoplasm (Fig. 2).

Macrostructure of the flocs

Aggregation experiments with latex beads showed flocs with different morphology and compactation depending on the presence/absence of ciliates. In presence of ciliates, few flocs (sometimes a

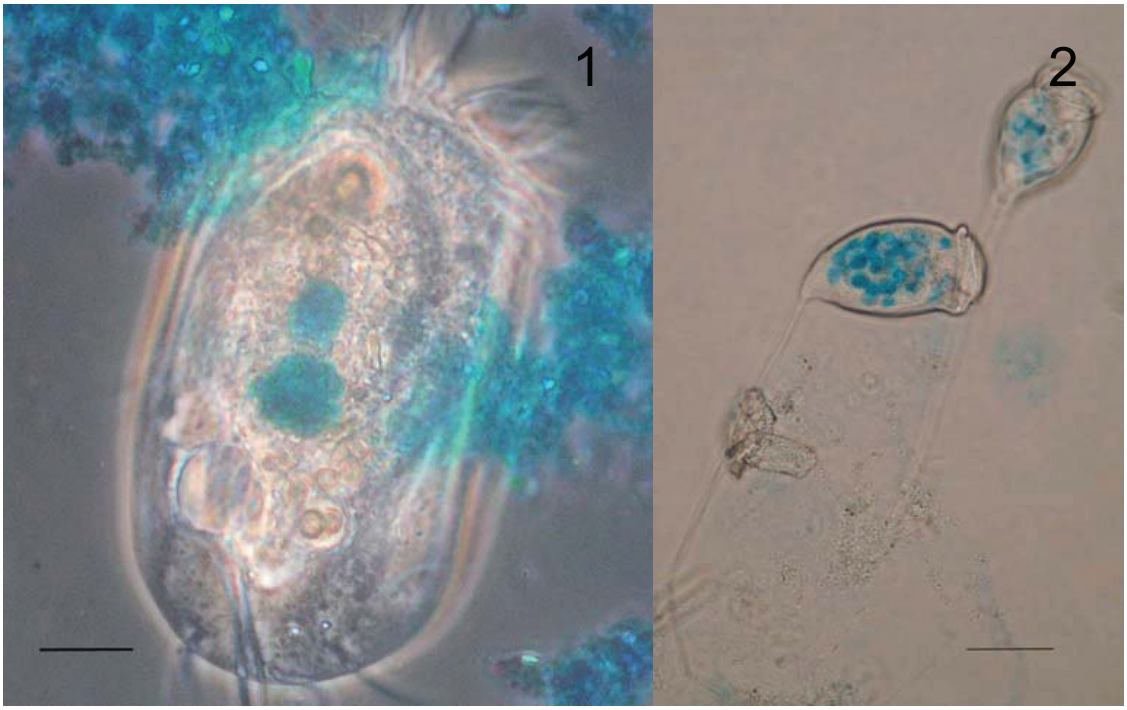

Figure 1, 2. Experiments performed with Alcian blue. **1**- Polymeric material secreted by *Euplotes* sp. **2**- Stained cytoplasmic vacuoles of *V. similis*. Scale bars = 20 μm.

unique floc) were observed with a defined morphology, compact (with few opened spaces) and a great cohesion among particles (Fig. 3).

Movements of crawling ciliates surrounding the floc and water flow due to the oral cilia beating of sessile ciliates tended to accumulate suspended particles towards these aggregates helping their adhesion. Vegetative cells of stalked *Vorticella* and cysts also contributed to floc formation (Figs. 4, 5).

Control experiments with bacteria however, showed numerous small aggregates, less compacted, with many opened spaces and less cohesion between particles than those found in the experiments with ciliates (Fig. 6).

Characterization of exopolymers

Characterization of carbohydrates, proteins and nucleic acids as components of the exopolymeric matrix of the flocs was carried out using different fluorochromes as specified in material and methods.

Carbohydrates

Con A (lectin from *Canavalia ensiformis*): *Euplotes* sp. cultures treated with Con A showed a matrix faintly stained with several small bright spots. Due to their shape and size these spots could be related to the bacteria employed as ciliate food (Fig. 7a). Those spots were found to be less abundant than in controls just with bacteria (Fig. 7b). *Vorticella similis* cultures showed certain parts of the matrix more intensively stained keeping latex particles closely packed among them. Groups of bacillary structures could be also visualized heterogeneously distributed within the matrix (Fig. 7c). Aggregates with cysts of *V. microstoma* showed a faintly stained matrix with areas of very intense fluorescence corresponding to cyst groups (Fig. 7d). This result shows the high affinity cysts walls had to this lectin, indicating presence of either D-glucose and/or D-manose within them.

PNA (lectin from *Arachis hipogaea*): In monoxenic cultures of *Euplotes* sp., bacillary forms were observed in a matrix weakly stained (Fig. 8a). These structures could be the bacteria *E. coli* employed as food. A higher bacterial density as described before was detected in the control experiment where the ciliate was absent (Fig. 8b).

On the other side, cultures of *V. similis* treated with PNA showed the presence of bacillary forms and amorphous material grouped in certain parts of the aggregates that were stained more intensively than in previous experiment (Fig. 8c). Results with

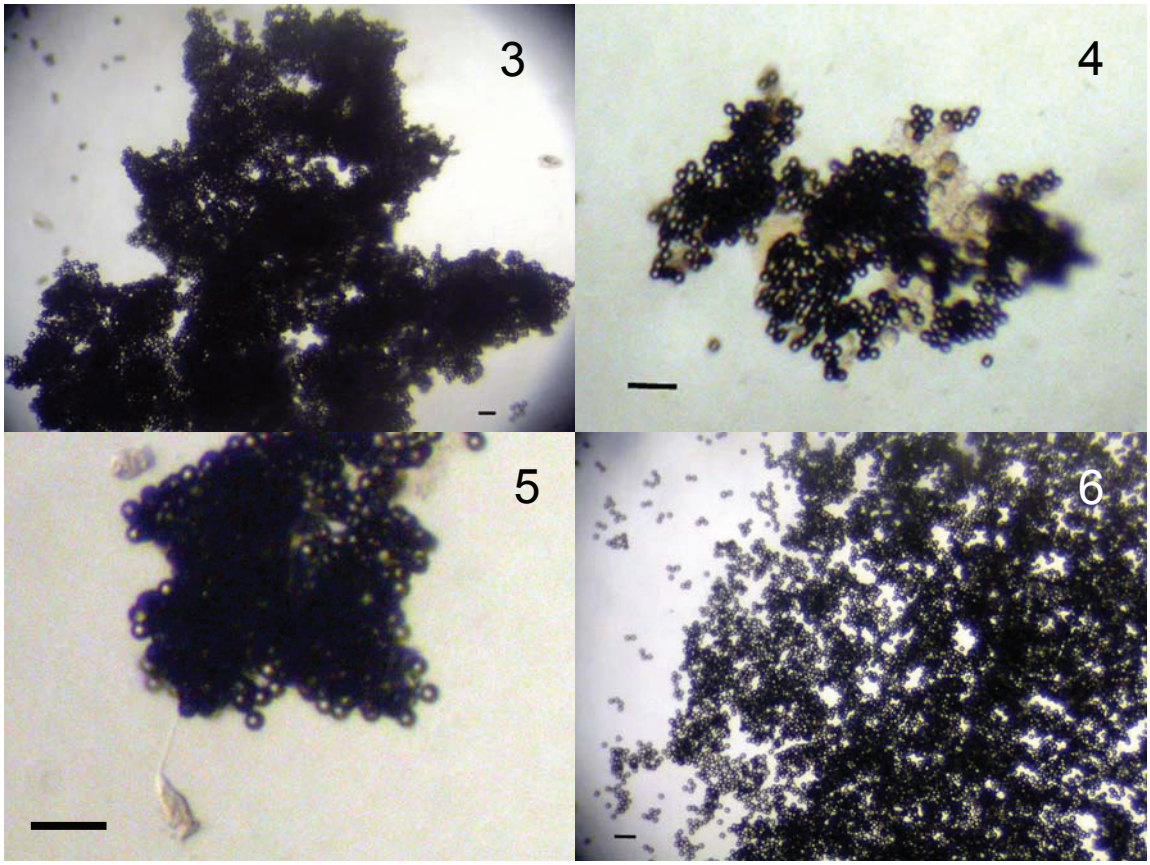

Figure 3–6. Aggregation of latex beads in cultures of *Euplotes* sp. (**3**), *V. microstoma* cysts (**4**) and *V. similis* vegetative cells (**5**). Note the presence of some specimens over or attached to the flocs. Control cultures (**6**). Scale bars = 100 μm.

cysts of *V. microstoma* were similar; the matrix, however, was more developed (Fig. 8d).

WGA (lectin from *Triticum vulgare*): In this case, *Euplotes* sp. and *V. similis* cultures showed analogous results: groups of bacteria appeared included in a matrix with brighter regions (Fig. 9a, b). Preparations with cysts of *V. microstoma* showed abundant regions intensively stained (Fig. 9c). Controls showed, as in other cases, the bacterial aggregates (Fig. 9d).

Proteins and nucleic acids:

DTAF staining for proteins from control experiments with *Euplotes* sp. was negative. However, *Vorticella* sp controls, showed bacterial cells weakly dyed with the fluorochrome.

Those experiments performed including bacteria or bacteria plus ciliates, showed bacillary shapes stained with DTAF surrounding the latex particles (Fig. 10a). These forms were clearly less abundant when ciliates were present (Fig. 10b). Cysts (Fig. 10c) and vegetative cells of *Vorticella* were also intensively dyed with DTAF, although stalks showed a weak fluorescence (Fig. 10d); in this case cells were observed embedded in a weakly stained matrix.

Propidium iodide used to detect nucleic acids showed non viable bacteria adhered to the latex particles and also included within a matrix that was stained as well (Fig. 11a). In the experiment adding ciliates, a brighter heterogeneous material was visualised linked to the particles and contributing to their cohesion (Fig. 11b). Nuclei of cells of the genus *Vorticella* were strongly stained with PI (Fig. 11c). A very strong fluorescence was also detected where cysts were located and this fact prevented a clear observation of their morphology (Fig. 11d).

Discussion

Crawling and sessile ciliates, members of the floc-associated biological community in activated sludge wastewater treatment plants, as shown in the experiments performed, contribute to bioaggregation. Aggregates produced in the presence of ciliates, as it has been demonstrated in these assays,

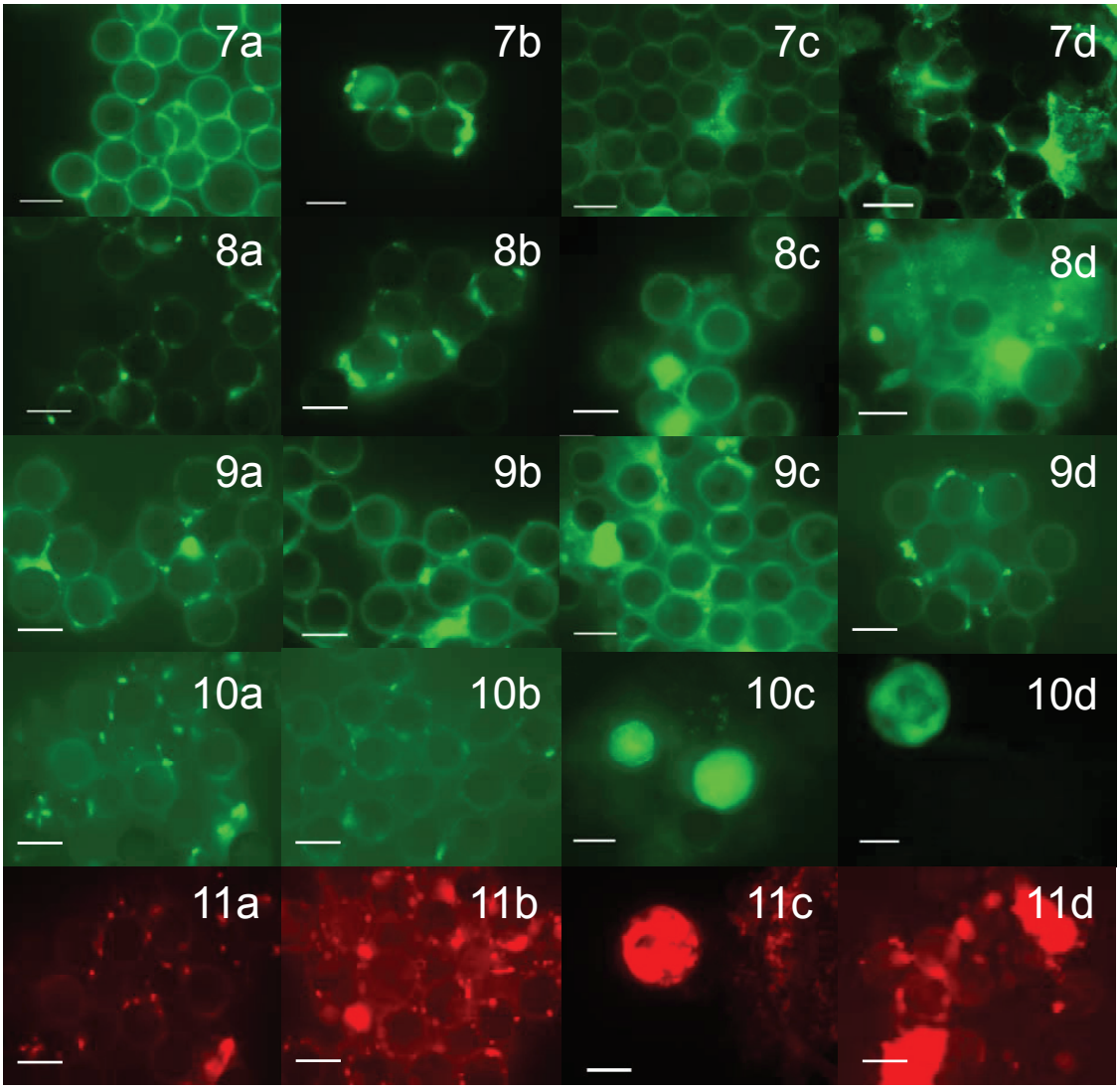

Figure 7–11. Aggregates of latex beads treated with different fluorochromes. **7**- Con A lectin, (**a**) *Euplotes* sp. (**b**) Control (**c**) *V. similis* (**d**) *V. microstoma* cysts. **8**- PNA lectin, (**a**) *Euplotes* sp. (**b**) Control (**c**) *V. similis* (**d**) *V. microstoma* cysts. **9**- WGA lectin, (**a**) *Euplotes* sp. (**b**) *V. similis* (**c**) *V. microstoma* cysts (**d**) Control. **10**- DTAF staining, (**a**) Control (**b**) Ciliate cultures (**c**) *V. microstoma* cysts (**d**) *V. similis*. **11**- PI staining, (**a**) Control (**b**) Ciliate cultures (**c**) *V. similis* (**d**) *V. microstoma* cysts. Scale bars = 20 μm.

were more compacted than those produced by bacteria alone.

Ciliates could contribute to aggregation by the active secretion of polymeric substances in several ways: (i) Extrusomes (ejectable membrane-bound organelles) are common in protozoan cells; their extrusion occurs after mechanical or chemical stimuli (for a review, Rosati and Modeo, 2003). (ii)Vesicles with an exocytotic function (ampules) have also been described in different species of *Euplotes* (Gliddon, 1966; Faure-Fremiet and André, 1968; Ruffolo, 1976; Dallai and Luporini, 1981; Görtz, 1982). These elongated vesicles are membrane-bound organelles, arranged close to the ciliary insertions (Fauré-Fremiet and André, 1968; Ruffolo, 1976). The nature of the ampule content is unclear, however our results suggest the existence of glycol-residues stained with alcian blue as it has been proposed by Görtz (1982). (iii) Cellular debris, excreted metabolic products, and non digested residues could also be involved in the aggregation of particles (Watson, 1945; Curds, 1963; Sleigh, 1979; Barker and Stukey, 1999; Laspidou and Rittmann, 2002). Excretion of all these compounds is the result of the microorganism growth, cell lysis and the interactions of the ciliates with their environment (Barker and Stuckey, 1999; Passow, 2002). The presence of these substances from ciliates within aggregates obviously facilitates their sedimentation capacity (Passow, 2002).

Other biological activities of ciliates could also play a role in the aggregation of particles, such as their movements around the floc and/or their feeding strategies (Sleigh, 1979). Several authors have demonstrated that ciliates are able to change the flux of water attracting nutrients towards the aggregates (Fried and Lemmer, 2003; Darbyshire, 2005). For example, the water flux produced by the oral cilia of peritrich ciliates is able to move particles to an extension of 400 µm. Besides, protozoa colonize biofilms since in this way they optimize their mechanisms for bacterial predation (Eisenmann et al. 1998). Many of them introduce part of the cell body within flocs interacting with the aggregate and detaching bacteria used for feeding (Darbyshire, 2005).

Cysts formation capacity of some ciliates also contributed to bioaggregation, as shown in the present study. These dormancy structures produced aggregates in presence or absence of latex beads. Resting cysts have thick mucous walls with a variable composition depending on the species. Its components are very often of polysacharidic nature (Hausmann et al. 2003) that exhibit flocculating properties. Other cell coverings, as loricas or shells have been also proposed as source of exopolymers to the environment (Sleigh, 1979).

Different types of microorganisms have been reported to produce EPS (Donlan, 2002). Among them glucose and galactose seem to be the most relevant sugar residues, although fructose, manose or glucuronic acid are also cited (Hu et al. 2003). Staining with lectins coupled to fluorescein (Con A, WGA and PNA) showed that the matrix over and between latex particles in our experiments contained glucose, manose, N-acetyl-glucosamine (NAG) and galactose. The abundant small bright spots observed could be identified as the short-rods of *E. coli* employed as food. Our results show that these enteric bacteria would be a source of NAG and galactose rests, although these residues seem to be restricted to structural elements of the cell surface as it has been described for *Pseudomonas aeruginosa* (Strathmann et al. 2002). WGA lectin would be linked to the LPS lipopolysacharide or any of the components of the bacteria mucous layer containing NAG or galactose. Other studies have also defined bacteria hydrated capsules as one of the EPS components (Gammar et al. 1997; Neyens et al. 2004).

The important adhesion capacity of proteins, mainly to hydrophobic substances, was pointed out by Donlan (2002), with hydrophobic interactions being of special importance. Proteins have high level of negatively charged aminoacids and they are more involved than sugars in the electrostatic binding with multivalent cations, which are important factors in the stabilization of aggregates (Laspidou and Rittmann, 2002; Görner et al. 2003). Bacteria are involved in exopolymeric matrix formation with an important protein contribution (van der Aa and Dufrêne, 2002). Several authors have suggested the proteins as main components of the EPS (Laspidou and Rittmann, 2002; Van der Aa and Duchêne, 2002; Görner et al. 2003) and their importance in the activated sludge of many biological reactors (Liu and Fang, 2002). Our results showed that protein fraction revealed over the latex beads surfaces could probably be of bacterial origin.

Cysts and zooids of *Vorticella* were strongly stained with DTAF, meanwhile stalk surface was faintly stained, so in this case only the ciliate cells would be an important protein source within the aggregate.

As inferred from our results with PI, the nuclei of ciliates are present in the polymeric matrix favouring particle aggregation. The genetic material seems to represent an important fraction of the EPS of ciliate source and, although the function of nucleic acids in the flocculation processes is not well known, these could contribute to support floc adhesion due to their negative charge.

Conclusions

In summary, the presence of ciliates associated to the floc -crawling and sessile ciliates- increases the efficiency of particle aggregation, which is essential for the good performance of an activated sludge wastewater treatment plant. Metabolism and biological activities of ciliates have to be taken into account as source of carbohydrates and nucleic acids to the matrix developed between particles. These compounds are essential for the proper establishment of aggregates; they stabilize interactions between cells and among cells and surfaces, giving rise to the typical architecture of flocs.

Acknowledgements

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