



METHODS TO EXTRACT THE EXOPOLYMERIC MATRIX FROM BIOFILMS: A COMPARATIVE STUDY

Joana Azeredo*, Valentina Lazarova** and Rosário Oliveira*

* *Centro de Engenharia Biológica – IBQF, Universidade do Minho, 4700 Braga, Portugal*

** *Centre of International Research for Water and Environment (CIRSEE), Lyonnaise des Eaux, 38 Rue du Président Wilson, 78230 Le Pecq, France*

ABSTRACT

To study the composition of a biofilm a previous extraction method is required to separate cells from the matrix. There are several methods reported in the literature; however they are not efficient or promote leakage of intracellular material. In this work several extraction methods were assayed in mixed culture and pure culture biofilms and their efficiency was evaluated by the amount of organic carbon, proteins and intracellular material extracted. The results showed that the extraction with glutaraldehyde 3% (w/v) was the most suitable method, extracting great amounts of organic carbon without promoting cell lysis or permeabilization. Glutaraldehyde is a bifunctional reagent that binds to cell walls avoiding their permeabilization and the biofilm matrix is solubilized in the solution. © 1999 IAWQ Published by Elsevier Science Ltd. All rights reserved

KEYWORDS

Biofilm; polymeric matrix; extraction methods; glutaraldehyde; cell leakage; exopolymers; proteins.

INTRODUCTION

To optimise the operating condition of a wastewater treatment plant, it is very important to determine the composition of biofilms, especially the number of microbial cells involved. The estimation of microbial cells has been made by means of determining the total protein content, dry weight of the total biomass, ATP or DNA (Lazarova and Manem, 1995).

Biofilms are formed by microbial cells embedded in an exopolymeric matrix (Charaklis and Wilderer, 1989). The polymeric matrix is mainly composed of polysaccharides, although other compounds can be found in smaller amounts such as proteins, DNA and humic substances (Nielsen and Jahn, 1996). So, the described above methods to determine the number of microbial cells do not give accurate values.

To estimate the accurate number of cells in a biofilm, a previous extraction method is required in order to separate microbial cells from the exopolymeric matrix. Several methods have been reported for extracting exopolymers from activated sludge flocs. These include high-speed centrifugation, steaming, ultrasonication and the use of chemical agents such as: NaOH or EDTA (Brown and Lester, 1980), Tris/HCl buffer (Goodwin and Foster, 1985) phosphate buffer and heat (Schmidt and Ahring, 1984), cation exchange resin (Frølund *et al.*, 1996) and formaldehyde (Fang and Jia, 1996). The most recent is the extraction with a

solution of glutaraldehyde (Azeredo *et al.*, 1998). As the structure of activated sludge flocs is not very different from biofilms, some of these methods have also been assayed in biofilms (Jahn and Nielsen, 1995).

Many of these methods have a very low efficiency in terms of selective exopolymer extraction and promote cellular lysis or intracellular material loss, which can distort the results (Azeredo *et al.*, 1998). In this work some of the above mentioned extraction methods were assayed in order to determine their ability to extract the polymeric matrix of biofilms. The extraction efficiency was determined by the yield in exopolymers and by the minimal effect on cell leakage.

MATERIAL AND METHODS

Samples

The extraction methods were assayed in two sewage biofilms (nitrifying and denitrifying) collected from a wastewater treatment plant in Evry, France (Lazarova *et al.*, 1998a) and in a biofilm produced by *Sphingomonas paucimobilis*.

Extraction methods

The assayed methods were: extraction with glutaraldehyde, Dowex resin, phosphate buffer and heating, Tris/HCl buffer and sonication. Prior to the extraction, the biofilms were washed with phosphate buffer (pH 7.0, 0.01M).

Extraction procedure

The extraction procedure and the analytical methods are depicted in Fig. 1.

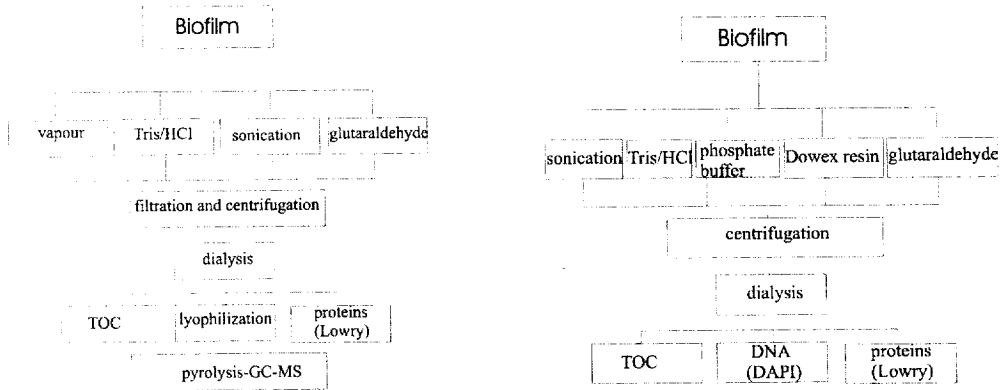


Figure 1. General extraction and analytical procedure for the nitrifying and denitrifying biofilms (left) and for the *Sphingomonas paucimobilis* biofilm (right).

Extraction with glutaraldehyde. 60 ml of glutaraldehyde 3% (w/v) were added to 20 g of the nitrifying biofilm (0.3425 mg of dry weight) and to 20g (0.292 mg of dry weight) of the denitrifying biofilm. 10 ml, 15 ml, 30 ml and 40 ml of glutaraldehyde 3% were added to 10 ml (75 mg of dry weight) of *Sphingomonas paucimobilis* biofilm. The biofilm suspensions were incubated overnight at 4°C under agitation (100 rpm).

Extraction with vapor. 20 g of the nitrifying biofilm and 20 g of the denitrifying biofilm were resuspended in 60 ml of phosphate buffer (pH 7.0, 0.01M). The biofilm suspensions were autoclaved at 120°C under 1 bar of pressure, for 10 minutes.

Extraction by sonication. 20 g of the nitrifying biofilm and 20 g of the denitrifying biofilm were resuspended in 60 ml of phosphate buffer (pH 7.0, 0.01M). A volume of 30 ml of phosphate buffer was

added to 10 ml of a *Sphingomonas paucimobilis* biofilm. The suspensions were sonicated for 1 min with a 13 mm probe (300 W sonicator, Bioblock), immersed 25 mm in the liquid, using a power output of 37 W. The tubes containing the samples were kept in crushed ice during sonication.

Extraction using a Dowex resin. 10 ml of the biofilm from *Sphingomonas paucimobilis* were resuspended in 30 ml of the extraction buffer (2 mM Na₃PO₄, 4 mM NaH₂PO₄, 9 mM NaCl and 1m M KCl). 30 g of Dowex resin (50X8, Na⁺ form, 20-50 mesh, Aldrich-Fluka 44445) previously washed with the extraction buffer were added to this suspension and the extraction took place at 400 rpm during 2 hours at -4°C.

Extraction with Tris/HCl buffer. 20 g of the nitrifying biofilm and 20 g of the denitrifying biofilm were resuspended in 60 ml of Tris/HCl buffer (pH 7.0, 0.1M). A volume of 30 ml of this buffer was added to 10 ml of *Sphingomonas paucimobilis* biofilm. The biofilm suspensions were immersed in a bath at 80°C during 1 hour.

Extraction using phosphate buffer. 10 ml of the biofilm from *Sphingomonas paucimobilis* were resuspended in 30 ml of phosphate buffer (pH 7.0, 0.01M). The biofilm suspensions were immersed in a bath at 70°C at 100 rpm during 2 and 4 hours.

The extracellular polymers were collected in the aqueous solution after centrifugation (9000 g during 20 min). The supernatants resulting from all the extraction methods were dialysed using a membrane of 14000 MWCO (Medicell, dialysis tubing-visking) against ultra-pure water for two days at 4°C.

Analytical methods

Total organic carbon (TOC). The samples were first acidified with phosphoric acid (final pH 2-3) and the inorganic carbon fraction was then eliminated by aeration. The total organic carbon was measured on a Beckman analyzer (Tocamaster™, Model 915B).

Total proteins. The total protein content was determined by the Lowry modified method, using the protein assay kit SIGMA P5656 with a standard of BSA (bovine serum albumin).

Pyrolysis-GC-MS. After freeze-drying, the extracted solutions were analysed by pyrolysis-GC-MS (Bruchet *et al.*, 1990). 1 mg of each sample was introduced into a quartz tube. The tube was inserted into a pyrolyzer filament and was heated to 650° C at a rate of 20° (ms)⁻¹. After pyrolysis, the fragments were separated on a 30 m DB WAX fused silica capillary column programmed from 20 to 400 amu at 1 scan s⁻¹.

Polysaccharides. The polysaccharides were determined by the Dubois *et al.* (1956) assay.

DNA. The DNA was determined using DAPI, according to Brunk *et al.* (1978).

RESULTS AND DISCUSSION

The extraction methods were assayed in three kinds of biofilms, two biofilms commonly used in waste water treatment and a biofilm produced by *Sphingomonas paucimobilis*, which is a great producer of gelane.

The efficiency of the extraction methods was evaluated by means of the ratio protein/total organic carbon (P/TOC) and the extent of cellular lysis was determined by the amount of DNA and intracellular components present in the extracted solutions. The intracellular material was determined by the furfuryl alcohol and acetamide residues obtained by pyrolysis-GC-MS (Lazarova *et al.*, 1998a).

The TOC and total protein content in the extracted solutions from the nitrifying and denitrifying biofilms are presented in Fig. 2. From these results it is apparent that vapour extracts the greatest amount of protein and TOC, giving a high ratio P/TOC (Table 1), which can indicate the occurrence of cell lysis. In a previous comparative study made with activated sludge it was found that this method promoted cell disruption (Azeredo *et al.*, 1998). Sonication enables the extraction of a very small amount of organic carbon and protein. The extract obtained with glutaraldehyde showed the smallest ratio P/TOC, followed by Tris/HCl.

This parameter can be an indicator of the extent of cell lysis or permeabilization, however it can not be used as a unique criterion (Azeredo *et al.*, 1998).

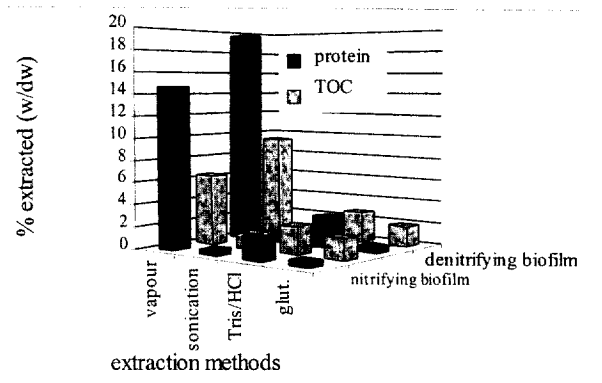


Figure 2. Total organic carbon and total protein extracted by sonication, vapour, Tris/HCl buffer and glutaraldehyde.

The results obtained by pyrolysis-GC-MS (Figure 3) show that with vapour 47% and 56% of intracellular substances were identified in the nitrifying and the denitrifying biofilm, respectively. The same results were obtained by Lazarova *et al.* (1998a), when assaying pyrolysis-GC/MS in the biofilm samples without a previous extraction step. This suggests that vapour promoted the extraction of all the biofilm content. The great amount of organic carbon extracted by Tris/HCl is probably due to some cell permeabilization. With this method 40% of the extracted solution are intracellular substances, mainly aminosugars, commonly found in cell walls. This fact can explain the small ratio P/TOC obtained by this method.

Table 1. Ratio protein/TOC measured in the extracted solution of nitrifying and denitrifying biofilms

Extraction methods	P/TOC	
	Nitrifying biofilm	Denitrifying biofilm
Vapour	2.28	3.06
Sonication	0.35	0.69
Tris/HCl	0.25	1.13
Glutaraldehyde	0.26	0.28

The small amount of organic matter obtained by sonication is mainly composed of proteins and intracellular constituents (Fig. 3). These two biofilms were strongly adhered to polymeric macroporous carriers (Lazarova *et al.*, 1998b), rendering difficult the action of the extraction methods. So, it seems that sonication only acted on the outer biomass, promoting cell leakage.

Glutaraldehyde was the agent that enabled the extraction of the smallest ratio P/TOC and the extracted solution was mainly composed of polysaccharides and humic substances. These substances are very common in wastewater biofilms (Jahn and Nielsen, 1995) and were only extracted using vapour and glutaraldehyde. It was shown that with vapour all the biofilm organic components are obtained and according to the pyrolysis-GC/MS results, about 50% is polymeric matrix. The amount of organic matter extracted with glutaraldehyde was about 22% of the organic carbon extracted by vapour and a minimum of intracellular material was obtained. As it has been mentioned, some intracellular material is always expected due to natural cell lysis.

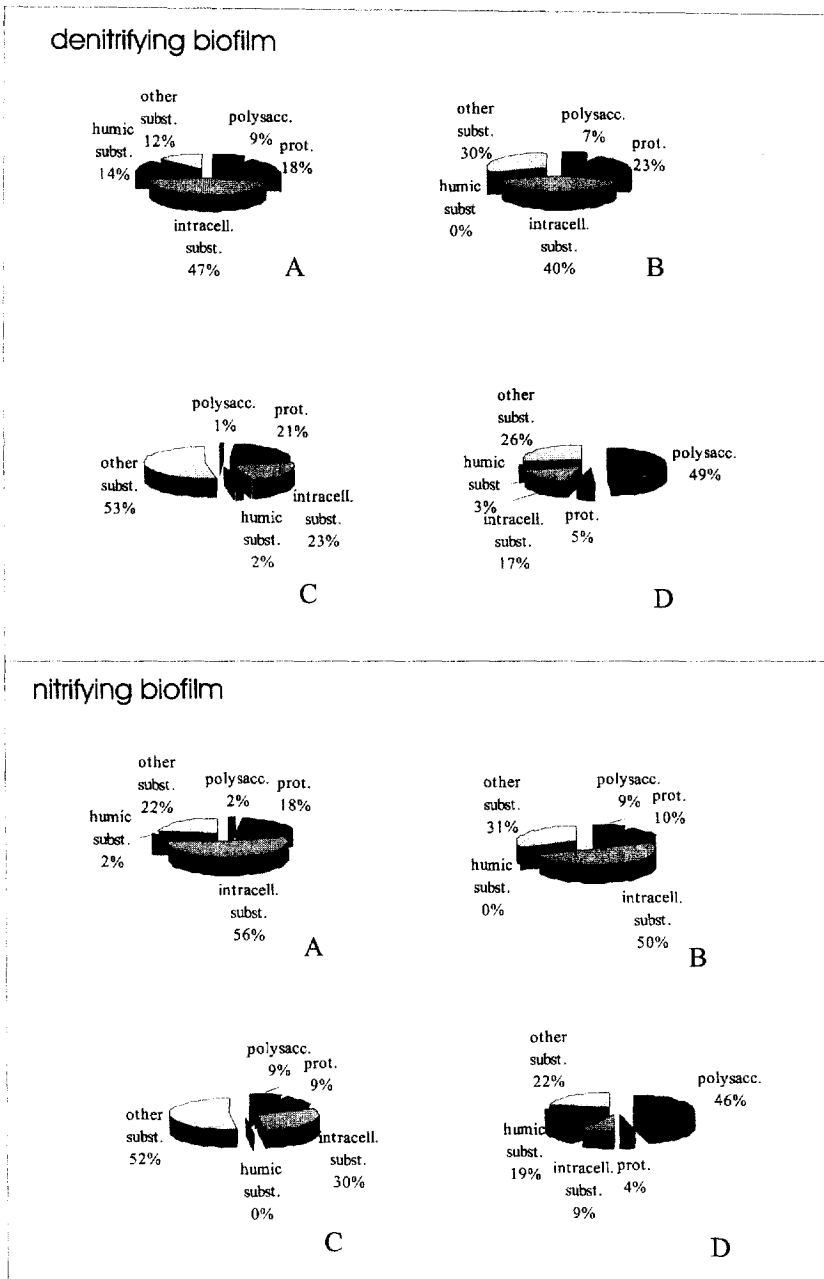


Figure 3. Results of the pyrolysis-GC-MS made on the extracted solutions obtained by vapour (A), Tris/HCl buffer, sonication (C) and glutaraldehyde (D).

The results of the extraction methods assayed in a *Spingomonas paucimobilis* biofilm (Fig. 4), show that the extraction methods that give the greatest amount of organic carbon are Dowex resin, Tris/HCl and glutaraldehyde. With phosphate buffer small amounts of protein and organic matter were extracted showing that this was not the most suitable method for this type of biofilm.

The extent of cell lysis was evaluated by the DNA content in the extracted solutions. This criterion was not used in the mixed culture biofilms because DNA can be found in sewage biofilms (Jahn and Nielsen, 1998). It was not very likely to find great amounts of DNA in the matrix of this pure culture biofilm because it was grown in a very rich medium and was a young biofilm (10 days) (Azeredo and Oliveira, 1998).

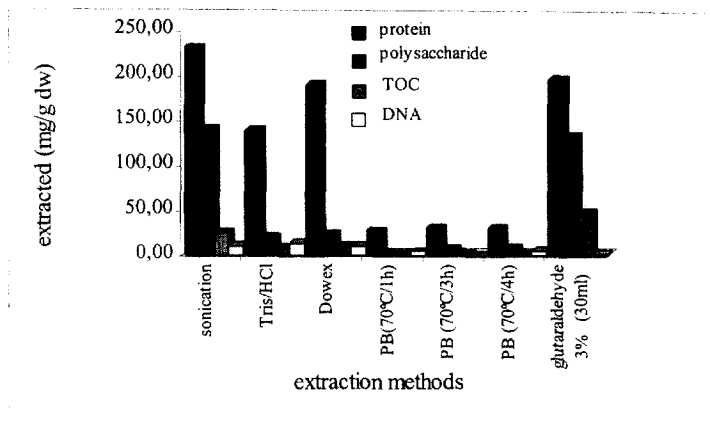


Figure 4. Extraction methods assayed in a *Sphingomonas paucimobilis* biofilm.

According to this criterion Tris/HCl, sonication and extraction with Dowex resin were aggressive to the cells. Therefore, part of the organic carbon extracted is a result of the release of some intracellular material. Glutaraldehyde enables the extraction of the greatest amount of polysaccharides and TOC and the smallest amount of DNA.

The efficiency of glutaraldehyde extraction is dependent on the amount of solution added (Fig. 5), but not on its concentration (Azeredo *et al.*, 1998).

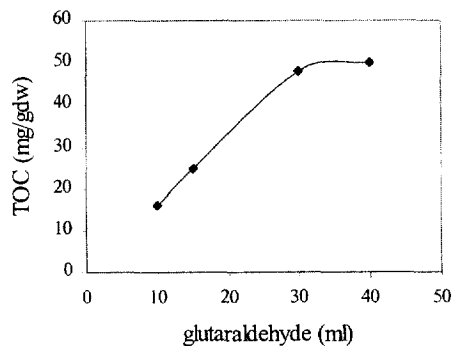


Figure 5. The influence of the volume of glutaraldehyde solution 3% (w/v) in the extraction efficiency of *Sphingomonas paucimobilis* biofilm.

The mechanism of glutaraldehyde extraction is not well understood, however it is known that this reagent is used to protect the cells against aggressive treatments, avoiding the deformation and permeabilization of the cell walls (Workman and Day, 1983). Moreover, microscopic observations of biofilms submitted to a treatment with glutaraldehyde showed the disappearance of the polymeric matrix that was formerly covering the cells (Fig. 6).

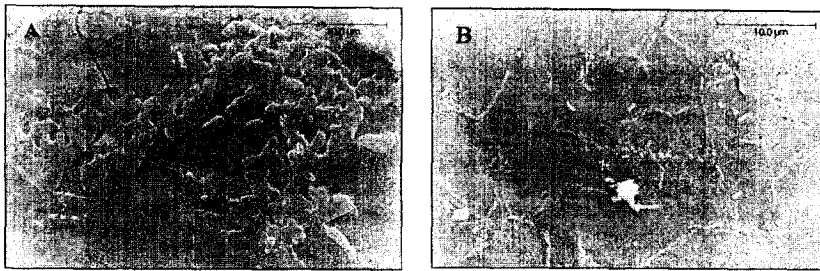


Figure 6. SEM observations of a nitrifying biofilm non-treated (A) and treated with glutaraldehyde 3% (B).

CONCLUSIONS

The criteria used to evaluate cell lysis must be carefully chosen. The ratio P/TOC is not the most suitable one and DNA identification can only be utilised in young biofilms. Other intracellular substances should be measured to determine the extent of cell leakage.

The extraction with glutaraldehyde was the method least aggressive to cellular biomass. This method enabled the extraction of about 22% of the total organic carbon of the sewage biofilms, where the polymeric matrix was very difficult to remove because the biomass was entrapped into macroporous carriers. Also in a pure culture biofilm, this method proved to be more suitable than the others, extracting the greatest amount of polysaccharides and TOC.

The other assayed methods exhibited small yields of extraction (phosphate buffer) or promoted cell lysis or permeabilization (vapour, sonication, Dowex resin and Tris/HCl buffer).

The yield of extraction with glutaraldehyde is dependent on the amount of solution added, so in each case it is necessary to optimise the extraction conditions.

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