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ORIGINAL PAPER

Assessment of physiological state of microorganisms in activated sludge with flow cytometry: application for monitoring sludge production minimization

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Abstract Many sludge reduction processes have been studied for the minimization of sludge production in biological wastewater treatment. The investigations on most of these processes have monitored the increase of the soluble chemical oxygen demand, the sludge mass reduction, or the decrease of the floc size, but little information has been obtained on cell lysis and the change of the biological cell activity. However, employing any strategy for reducing sludge production may have an impact of microbial community in biological wastewater treatment process. This impact may influence the sludge characteristics and the quality of effluent. The objective of this study concerns the determination of the physiological state of activated sludge microorganisms during a sludge minimization process. A thermal treatment at 80 °C for 5, 20, 40 and 60 min was chosen in this study. Staining bacteria with CTC and SYTOX green was used to evaluate biological cell activity and viability of cell types contained in activated sludge, respectively. The monitoring of cell activity and viability was performed using flow cytometry (FCM) analysis before and after thermal treatment of activated sludge. Results indicated an increase in the number of permeabilized cells and a decrease in the number of active cells, subsequent to the thermal treatment. The study also confirms the potential of FCM to successfully evaluate the physiological heterogeneity of an activated sludge bacterial population. Moreover, the experimentally observed correlations between the FCM results and the organic matter solubilization in activated sludge samples during thermal treatment revealed that the increase in the soluble organic matter concentration was predominantly due to an intracellular material release. Identifying the increase in activated sludge hydrolysis requires a precise knowledge of the involved mechanisms, and this study indicated that the FCM, used in conjunction with specific probes, could be a useful tool.

Keywords Activated sludge · Flow cytometry · Cell lysis · Respiratory activity · Hydrolysis treatment

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Introduction

Considerable research has been devoted in the past decades to the optimization and control of biological wastewater treatment processes. Many treatment processes have been studied to increase the methane potential of sludge with a rate limiting hydrolysis stage of organic matters associated with microbial cells [1, 2]. Sludge hydrolysis could be enhanced by physical (thermal or mechanical) [3–5], chemical (using acids or alkali) [6], or combined treatments (thermo-chemical, chemo-mechanical etc.) in order to improve sludge digestion and thus to reduce waste sludge. Among these processes, thermal treatment allows for significant reduction of excess sludge production [7, 8]. For thermal treatment, two main temperature ranges are to be considered; the processes that take place under 100 °C and under normal pressure, and processes that take place

above 100 °C in which a pressure reactor is needed. While much stronger effects are reported in the case of high temperature/pressure treatment, these processes are costly and require maintenance. On the other hand, thermal treatment at temperatures lower than 100 °C applied to a conventional biological system (activated sludge) allows for significant reduction of excess sludge. Indeed, a 15% increase in methane production was demonstrated by Li and Noike [9] at a treatment temperature of 80 °C. However, whatever the temperature range, when sludge heating is performed in order to reduce sludge production, sludge solubilization will be required. Also, to be optimal this hydrolysis step should include both a reduction of the particulate organic matter into lower molecular weight compounds, and cell lysis. Since very few studies characterised the biological effect of temperature on sludge disintegration, especially in the case of temperature below 100 °C, there is a need for data to understand by which mechanism these treatment act on sludge.

The majority of the sludge reduction studies have focused either on the increase in the soluble chemical oxygen demand (SCOD), the sludge mass reduction, or the decrease in the floc size, but little information has been obtained about cell lysis or changes in the biological activity [1, 2]. The characterization of impact of enhanced hydrolysis by the pretreatments mentioned above in terms of microbial activity (active cells able to convert organic matter) and viability (cell lysis with the resulting release of intracellular material) is fundamental to sludge reduction optimisation. Enumeration of microorganisms present in the activated sludge by the conventional culture techniques of microbiology such as viable plate count or most-probable-number is one of basic analysis towards the microbial activity and viability assessment. Unfortunately, most environmental microorganisms are unable to be cultured on general media used in laboratories. Consequently, alternative methods for analysis of bacterial functions have been developed. Wallner et al. [10] studied the correlation between microbial community structure and function in activated sludge using the in situ identification of microorganisms with rRNA-targeted probes in combination with flow cytometry (FCM). More recently, Ziglio et al. [11] developed a procedure to disaggregate sludge flocs before staining (with dyes) and FCM analysis and demonstrated that fluorescent dyes combined with FCM can be a tool for assessment of viability and activity of an activated sludge mixed bacterial population. Andreottola et al. [12] also used fluorescent dyes combined with FCM to measure the viable fraction of particulate COD in activated sludge samples. These studies indicated that when used in conjunction with fluorescent probes, FCM allows a rapid and accurate quantification of the total bacterial population, including the viable but not culturable fraction.

Various fluorescent probes can be used for the assessment of bacterial viability and activity [13–16]. Metabolic activities may be measured with tetrazolium compounds such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC), which is reduced by respiring microorganisms to yield a fluorescent formazan precipitate [17]. An alternative approach relies on the fluorescence-based detection of the electrical potential gradient across the bacterial plasma membrane monitored by the accumulation of positively charged dyes such as rhodamine 123. Enzyme activity could also be detected with a variety of fluorogenic substrates such as fluorescein diacetate (FDA) or carboxyfluoresein diacetate (CFDA). Similarly, membrane cell damage could be detected by the use of cellular integrity dyes such as propidium iodide (PI) or SYTOX Green (SG) [18]. These indicators only diffuse cells with permeabilized membranes and exhibit marked fluorescence enhancement within the cells. All these measurements (enzyme activity, redox potential, and membrane integrity) can provide very specific and useful information about the physiological state of bacteria.

Whereas it's well known that FCM could be a relevant tool in environmental studies, it is not usually used to monitor biological changes during sludge reduction processes. This study focuses on the possibility of using FCM to evaluate the physiological state changes of bacteria occurring during sludge thermal treatment. In this paper, the applicability of FCM in combination with fluorescent probes to assess microorganism viability and activity in activated sludge was investigated. In parallel, the determination of organic matter solubilization by COD, protein and sugar assays was also performed in order to have a better understanding by which mechanism thermal treatment acts on sludge.

Materials and methods

Activated sludge characteristics

Experiments were carried out on an activated sludge sample collected from a municipal wastewater treatment plant (WWTP) in Limoges (France). The plant has a capacity of 285,000 people equivalent and it treats domestic wastewater. The activated sludge unit in the WWTP has hydraulic and sludge retention times of 8 h and 12 days, respectively. The activated sludge sample, collected in the aerobic zone, had a total suspended solids (TSS) concentration of 3.5 g/L.

Heat treatment

Experiments were carried out in glass volumetric flasks containing 0.4 L of activated sludge. Samples were placed

in a water bath (Isotemp, Bioblock Scientific, France) at 80 ± 0.2 °C. Thermally pre-treated sludge samples had different holding times (5, 20, 40 and 60 min at 80 ± 0.2 °C) in the water bath. The flow chart for the analytical procedure is shown in Fig. 1.

Microbiological studies

Activated sludge sample disaggregation

Activated sludge samples (0.3 L) were disaggregated with a mechanical blender (Ultra-Turrax-T25; IKA Labortechnik, Germany) at 24,000 rpm for 10 min. After pre-treatment, samples were filtered through a 48 μm pore-size membrane in order to avoid clogging of the cytometer nozzle. The filtered activated sludge samples were then diluted with a phosphate-buffered saline solution (prefiltered through 0.22 μm) in order to give a final concentration of approximately 10^6 microorganisms/mL. Two parameters were then monitored: the total number of cells detected with FCM after staining of samples using the Bacteria Counting Kit® as well as the total number of culturable cells using the plate count method.

Fluorescent probes and cell staining protocol

Three staining procedures based on the individual use of the Bacteria Counting Kit® (Invitrogen, France), SYTOX Green (Invitrogen, France) and CTC (Sigma, France) were performed.

The Bacteria Counting Kit® was used to determine the total bacterial cell numbers of sludge samples. The staining procedure was performed as recommended by the manufacturer. A volume of 0.5 μL of the dye was added to 500 μL of disaggregated and filtered sample and was incubated for 5 min at room temperature (20 ± 2 °C). Microsphere suspension was resuspended by sonication in a water bath for about 5 min. After sonication 5 μL of the microsphere suspension was added to the SYTO BC-stained cell preparation before FCM analysis in order to have a final microsphere concentration of approximately 10^6/mL.

SYTOX Green, a cell impermeant fluorescent probe, was used for damaged and dead cell detection. It was delivered in dimethyl sulfoxide at a concentration of 5 mM. This commercial stock solution was stored at −20 °C. SYTOX Green was added to samples at a final concentration of 5 μM. Samples were then incubated for 5 min at room temperature as suggested by the manufacturer before FCM analysis.

Respiring bacterial cells were stained with CTC. The CTC stock solution was added to pre-treated (thermally or mechanically) activated sludge samples at a final concentration of 2 mM. Samples were incubated for 30 min at 37 °C in dark conditions without shaking [19].

Flow cytometric (FCM) analysis

The analysis were performed using a FACS Vantage cell sorter (Becton Dickinson, MD, USA) equipped with a 488 nm argon laser (excitation wavelength of SYTO BC, SYTOX green and CTC). Three parameters were measured: Forward-angle light scatter (FSC) related to cell size, green fluorescence stemming from SYTO BC or SYTOX Green, and red fluorescence originating from CTC. Two band pass filters of 530 and 610 nm were used to collect the green (FL1) and the red fluorescence (FL3) respectively. FSC was collected with a diode detector. The green fluorescence signals were collected with photomultiplier tubes. FSC, FL1 and FL3 were collected in 4 decades logarithmic scale. Approximately 10,000 cells were analyzed at a flow rate of 500 cells/s. Data were analyzed with the free software WinMDI version 2.9 (Scripps Research Institute).

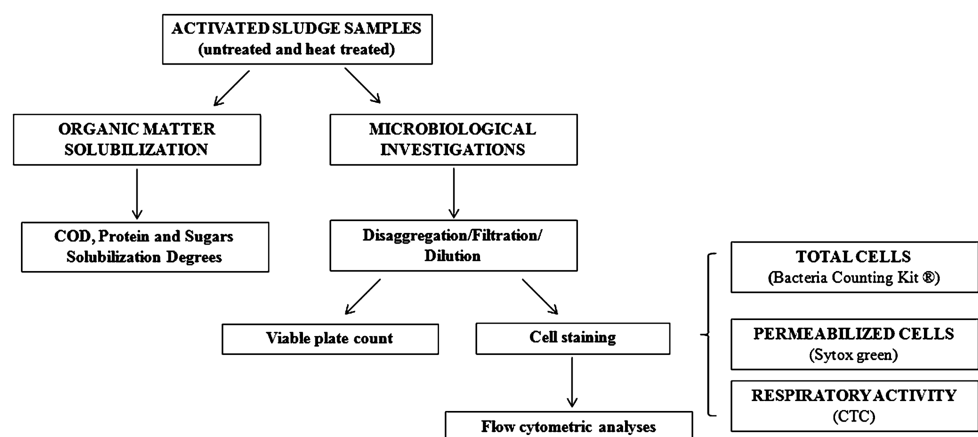

Fig. 1 Flow chart of the analytical procedure

For cell concentration determination using FCM analysis, the data indicated are mean of duplicates. In this case the standard deviations (SD) were not significant enough to be reported, due to the large number of events analysed.

Measurement of cell culturability

Culturability of bacteria from disaggregated and filtrated samples was evaluated by plate counts and compared with FCM results. After serial dilutions in a sterile phosphate-buffered saline solution, 1.0 mL aliquots of the dilutions were inoculated into aerobic plate count agar (Difco, Detroit, USA) containing 5 g/L of pancreatic digest of casein, 2.5 g/L of yeast extract, 1 g/L of dextrose and 15 g/L of agar. Each dilution was spread in duplicate. Colony forming units (CFU) were then determined after incubation at 37 °C for 48 h. Each result was the arithmetic mean of duplicates.

Solubilization evaluation

Raw and heat treated sludge samples were centrifuged at 6,000*g* for 20 min. Supernatants containing the soluble fraction of sludge underwent a 0.45 μm pore-size filtration in order to eliminate any residual biomass and other particulate matter. For each raw and heat treated sample, the total sludge and the filtered supernatant were characterized for COD, protein, and sugar concentrations.

To determine protein concentrations, the method proposed by Lowry modified by Frolund et al. [20] was used. Bovine serum albumin (BSA, Sigma, France) was used as the protein standard. Concentrations of sugars were determined by the method of Dubois et al. [21]. Glucose solution was used as the sugar standard.

COD concentrations were measured according to the Hach method (HACH, 1996–2000) [22]. COD solubilization degree, which represents the transfer of COD from the particulate fraction to the soluble fraction of the sludge during heat treatment [4], was expressed according to the equation:

$$\text{COD solubilization}\,(\%) = \left[(\text{COD}_s - \text{COD}_{si}) / \text{COD}_{pi}\right] \times 100 \quad (1)$$

where COD_{si} and COD_s were the soluble COD measured in the filtered supernatants initially (before heat treatment) and after heat treatment, respectively. COD_{pi} was the initial (before treatment) particulate COD.

Biopolymer solubilization (transfer of protein and sugars, which are the principal components of biopolymers, from the particulate fraction to the soluble fraction of the sludge) was determined in the same manner:

$$\text{Protein solubilization}\,(\%) = \left[(\text{proteins}_s - \text{proteins}_{si}) / \text{proteins}_{pi}\right] \times 100 \quad (2)$$

$$\text{Sugar solubilization}\,(\%) = \left[(\text{sugars}_s - \text{sugars}_{si}) / \text{sugars}_{pi}\right] \times 100 \quad (3)$$

The particulate fraction corresponds to the solid phase after centrifugation. Consequently, particulate concentrations for COD, proteins and sugars were obtained by the difference between the total and soluble concentrations.

Results were reported from duplicate determination for each sample. The data indicated are mean of duplicates ± SD.

Results and discussion

Application of FCM to determine cell number in activated sludge

The total number of cells was determined using FCM on activated sludge samples after staining with the Bacteria Counting Kit®. The basis of the Bacteria Counting Kit® procedure is to stain microorganisms selectively with a DNA fluorescent probe, the SYTO BC, and to mix the labelled bacteria with a known concentration of calibrated fluorescent beads. The FCM produced a FSC (related to cell size) versus SYTO BC green fluorescence cytogram (Fig. 2) in which it was possible to distinguish three areas: (1) the DNA containing cells with a typical amount of

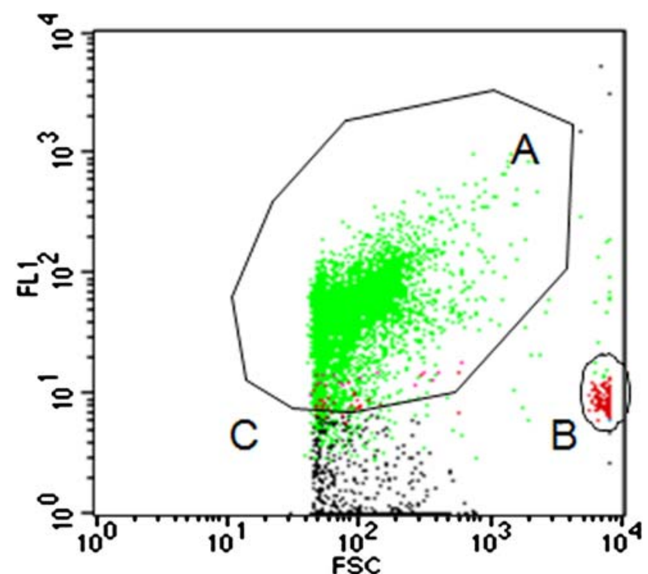

Fig. 2 FSC versus green fluorescence cytogram for activated sludge cells stained with the Bacteria Counting Kit®. The diagram refers to a total count of about 10,000 cells

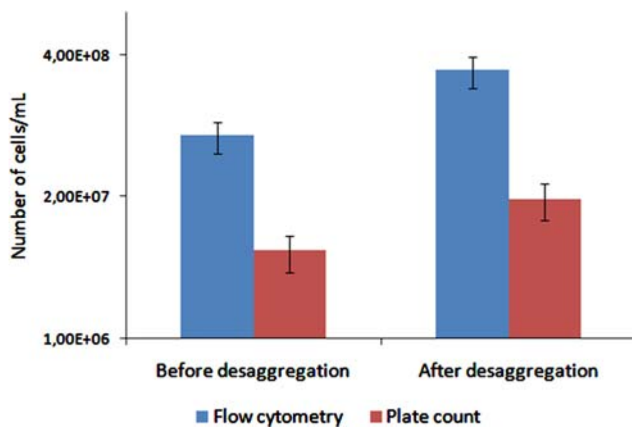

Fig. 3 Effect of the disruption procedure on both total cell concentration determined by FCM and culturable cell concentration determined by the plate count method (data indicate mean of duplicates ± SD)

DNA and normal cell size (A), (2) the fluorescent bead population (B), and (3) the noise and background with autofluorescent particles of organic matter and debris stained by unspecific binding of the fluorescent dye (C).

The addition of a known final concentration of fluorescent beads in sludge samples allowed for the evaluation of total number of microorganisms according to the Eq. (4):

$$\text{Number of bacterial cells} = (\text{bacteria events}/\text{beads events}) \times \text{beads number} \times \text{dilution factor} \tag{4}$$

In order to apply Eq. (4) to determine the microorganisms present in activated sludge, a preliminary mechanical disruption procedure was required. In a complex activated sludge matrix, the majority of microorganisms are aggregated into flocs which need to be disrupted prior to FCM analysis. For this, a mechanical disruption procedure, as previously reported by Ziglio et al. [11] was applied. In order to ensure that the disruption protocol did not have deleterious effects on microorganisms, the culturable cell concentration was also established (Fig. 3). As shown in the Fig. 3, the total number of cells was significantly greater in disaggregated samples compared to untreated ones. After the mechanical disaggregation, the total individualized cell number was multiplied by 4. The culturable cell concentrations obtained by plate count were lower than those obtained by FCM, since the Bacteria Counting Kit® stained all the cells (live and dead). But the results showed the same trend, that is, the culturable cell concentration increased after disaggregation.

These results indicated that the mechanical treatment achieved disruption of bacterial cells in an activated sludge with a minimal damage to the microorganisms. Although we can not exclude the possibility that disaggregation may induce damage to a portion of the bacterial cell population, these results show also that mechanical disruption does not generate drastic damage to microorganisms, since the culturable cell number is higher before as compared to after disaggregation. Similarly, Ziglio et al. [11] reported a significant increase in cell concentration after a mechanical disruption procedure performed in two steps, without any effect on cell membrane integrity. Falcioni et al. [23] compared sonication and mechanical homogenization as treatment for activated sludge cell individualization. They concluded that mechanical disruption showed a good linearity in results with respect of cell viability.

Physiological state evolution of microorganisms during the thermal treatment

Table 1 displays the results of total cell number evolution of activated sludge microorganisms during the thermal treatment with the use of FCM in conjunction with fluorescent dyes. As it can be seen from Table 1 the total cell number remained nearly constant during thermal pretreatment. However, the results took into account all the bacteria, including both live and dead, since the SYTO BC-stained all the cells. So, in order to have a better understanding of the real cell modifications induced by the thermal treatment, cell viability and activity were also investigated.

Membrane integrity was studied using the SYTOX Green. SYTOX green is a DNA binding probe that diffuses only into the cells with a damaged membrane. After diffusion into injured cells, its DNA binding results in a green fluorescence emission. Green fluorescence histograms of the different samples obtained after FCM analysis are presented in the Fig. 4. A clear separation between stained and unstained subpopulations was obtained after FCM analysis. This indicated that although the total cell concentrations remained constant (Table 1), viability changes occurred during heating. The fact that the green fluorescence intensity increased as higher SYTOX green diffused

Table 1 Cell concentrations detected by FCM analysis before and after different times of 80 °C thermal treatment (data indicate mean of duplicates)

Samples	Control (untreated)	80 °C, 5 min	80 °C, 20 min	80 °C, 40 min	80 °C, 60 min
Cell concentrations (Bacteria/mL)	1.11E+08	1.28E+08	1.52E+08	2.06E+08	2.02E+08

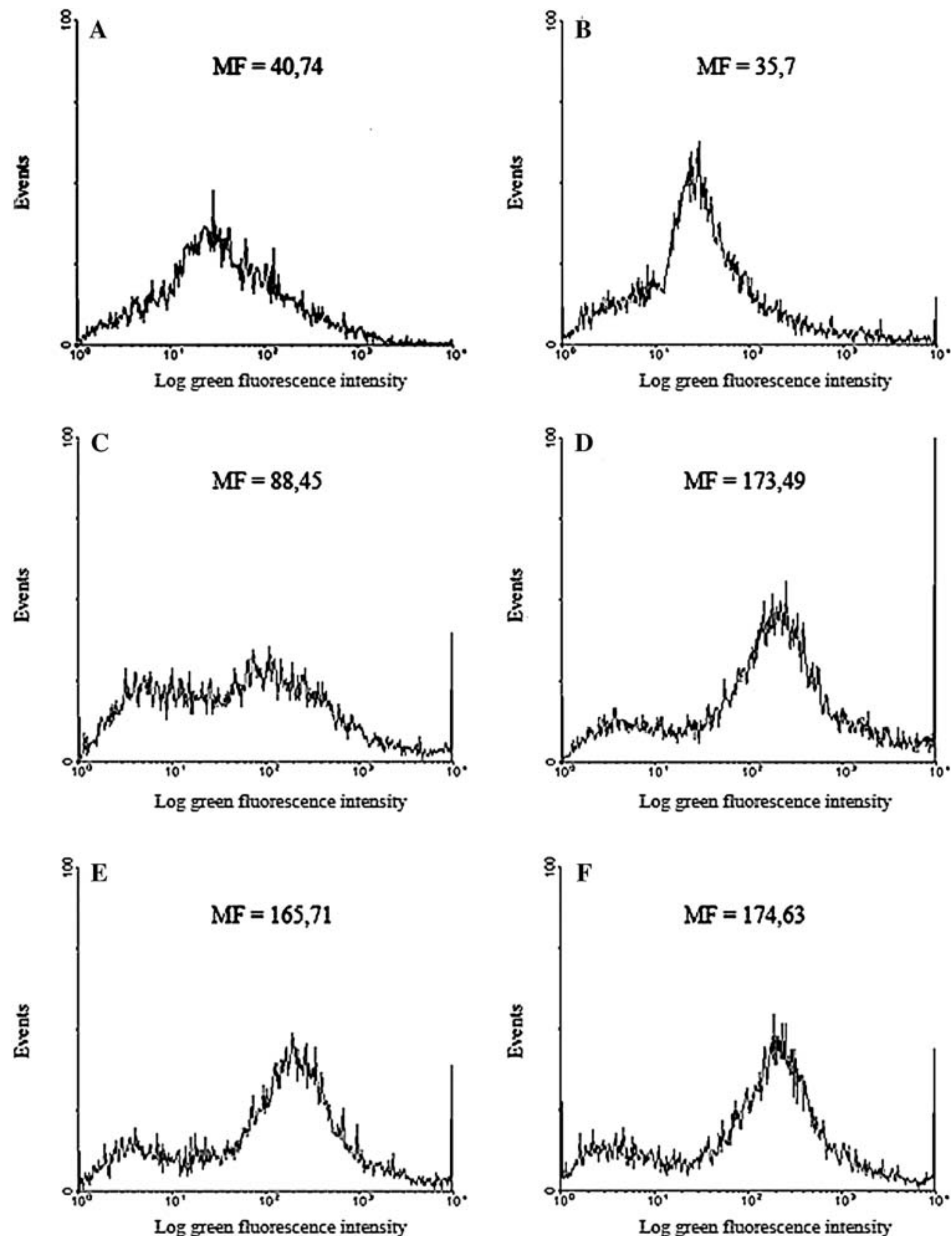

Fig. 4 Green fluorescence histograms of: untreated (control) before (**a**) and after (**b**) the disruption procedure and heat treated at 80 °C for 5 min (**b**), 20 min (**c**), 40 min (**d**), and 60 min (**e**) activated sludge cells stained with the SYTOX Green. The diagrams refer to a total count of about 10,000 cells

into the cells during heat treatment indicated a cell damage as the treatment time increased (Figs. 4, 5). The histogram profiles obtained on the bacterial cell population before (Fig. 4a) and after (Fig. 4b) the disruption procedure were rather similar. Such results support the finding that the mechanical disaggregation induced no significant deleterious effects on microorganisms.

In parallel to membrane integrity, respiratory activity was also studied using a tetrazolium salt, the CTC. This fluorescent dye could be used as an artificial electron acceptors to detect dehydrogenase activity. This probe reduction by active cells resulted in a red fluorescence emission. As expected, the results obtained after FCM analysis of CTC stained samples displayed an inverse relationship with the SYTOX Green fluorescence intensity. A decrease in the red fluorescence intensity, due to the loss of respiratory activity, was indeed observed as the treatment time increased (Fig. 5). These findings indicated that heating induced concurrently a loss of both membrane integrity and respiratory activity which were confirmed by the results obtained by plate count, which showed a loss of culturability of microorganisms after the thermal treatment. These results confirmed that the FCM, used in conjunction with specific probes, could be used for a rapid assessment of physiological states of microorganisms in activated sludge.

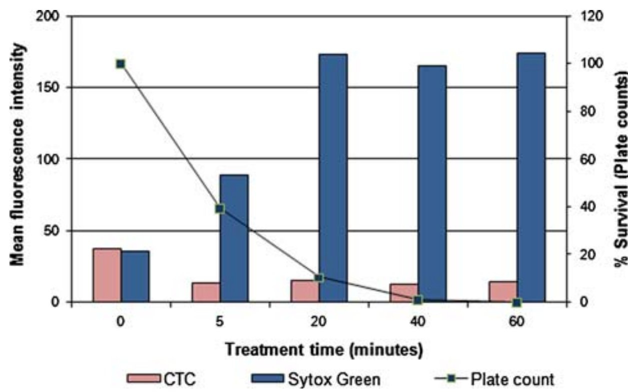

Fig. 5 Viability assessment of heat treated activated sludge cells by FCM and plate counts. Results were expressed as the mean of fluorescence intensity and the percentage of survival as determined by plate counts (in relation to the control)

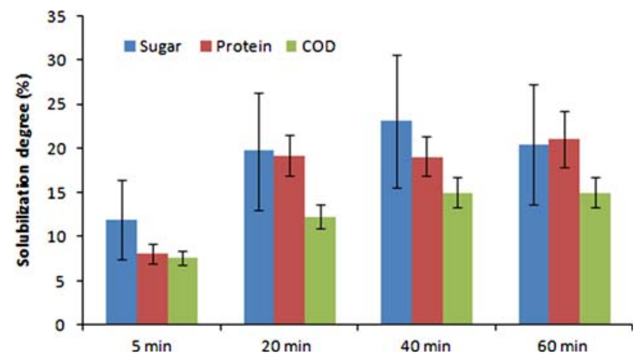

Fig. 6 COD, protein and sugar solubilization degree evolution during a thermal treatment (data indicate mean of duplicates ± SD)

Effects of thermal treatment on organic matter solubilization

The sludge minimization processes, such as thermal treatment, convert the particulate organic matter into soluble organic matter that can more easily be assimilated by the active biomass. This transformation is performed, in theory, firstly by cell lysis and secondly by reducing the size of the organic matter molecules. So, in parallel to the monitoring of biological changes induced by the thermal treatment, the transformation of the particulate organic matter into soluble organic matter was also evaluated. In this study, the solubilization degree for COD, proteins and sugars were determined according to Eqs. (1), (2) and (3), respectively and the results are presented in Fig. 6. As it can be seen from Fig. 6 the thermal treatment at 80 °C was successful in releasing COD, proteins and sugars from the particulate phase of activated sludge into the soluble phase. Although, the solubilization degrees for all COD, proteins and sugars increased with treatment time until 20 min, no additional effects were observed for particulate organic matter solubilization above this exposure time. These results are in agreement with previous studies which reported that matter and COD solubilization increased with temperature, but treatment time had little effect if it exceeds 30 min in a temperature range of 60–190 °C [24]. Similarly, in another study [25], sludge COD solubilization rate dramatically decreased after 30 min of thermal treatment.

Moreover, when solubilization results were compared with those obtained with the FCM analysis, a clear correlation between cell lysis and organic matter solubilization could be observed. The green fluorescence intensity, related to cell lysis, increased until 20 min of thermal treatment, and remained constant beyond this time exposure (Figs. 4, 5). The relation between cell lysis and organic matter solubilization implies that the origin of the released organic matter during the thermal treatment was mainly intracellular. This hypothesis is in accordance with Paul et al. [25] who concluded that a thermal treatment below 100 °C allowed only partial floc sludge destruction. These treatment conditions should be sufficient to break the non-covalent links and modify the sludge structure, but are insufficient to allow a complete floc destruction. Therefore, after the thermal treatment, for the experimental conditions tested in this study, it seems that the major part of soluble organic matter originated from intracellular material release, due to cell lysis, rather than extracellular polymeric network.

Conclusion

In this paper, the possibility of using FCM in combination with fluorescent probes to assess bacterial viability in activated sludge was investigated. Furthermore, FCM combined with fluorescent probes was also applied to monitor biological changes during thermal hydrolysis concurrently to the organic matter solubilization. Based on the experimental data and analysis the following conclusions are drawn:

1. The mechanical disruption procedure applied in order to disrupt the floc structure separated cells without major damage to the microorganisms, since the total cell concentration assessed by FCM as well as the culturable cell concentration increased after disaggregation.
2. The use of fluorescent dyes combined with FCM analysis provides a way not only to distinguish live and dead activated sludge cells but also to discriminate between different physiological states of a stress population during a thermal treatment.
3. The relationship between the FCM results and the organic matter solubilization degrees during activated

sludge heating seemed to demonstrate that the increase in the soluble organic matter concentration was predominantly due to an intracellular material release. The improvement of activated sludge hydrolysis treatments requires a precise knowledge of the involved mechanisms. This study showed that FCM, used in conjunction with specific probes, could be a relevant tool to determine precisely the origin of the released soluble organic matter after treatment.

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References

1. Liu Y, Tay JH (2001) Strategy for minimization of excess sludge production from the activated sludge process. Biotechnol Adv 19:97–107
2. Wei Y, Van Houten RT, Borger AR, Eikelboom DH, Fan Y (2003) Minimization of excess sludge production for biological wastewater treatment. Water Res 37:4453–4467
3. Eskicioglu C, Kennedy KJ, Droste RL (2006) Characterization of soluble organic matter of waste activated sludge before and after thermal pretreatment. Water Res 40:3725–3736
4. Bougrier C, Delgenès J-P, Carrère H (2006) Combination of thermal treatments and anaerobic digestion to reduce sewage sludge quantity and improve biogas yield. Process Saf Environ Prot 84:280–284
5. Gronroos A, Kyllonen H, Korpijarvi K, Pirkonen P, Paavola T, Jokela J, Rintala J (2005) Ultrasound assisted method to increase soluble chemical oxygen demand (SCOD) of sewage sludge for digestion. Ultrason Sonochem 12:115–120
6. Rocher M, Goma G, Pilas Begue A, Louvel L, Rols JL (1999) Towards a reduction in excess sludge production in activated sludge processes: Biomass physicochemical treatment and biodegradation. Appl Microbiol Biotechnol 51:883–890
7. ØDegaard H (2004) Sludge minimization technologies: an overview. Water Sci Technol 49:31–40
8. Pérez-Elvira SI, Nieto Diez P, Fdz-Polanco F (2006) Sludge minimisation technologies. Rev Environ Sci Biotechnol 5:375–398
9. Li YY, Noike T (1992) Upgrading of anaerobic digestion of waste activated sludge by thermal pretreatment. Water Sci Technol 26:857–866
10. Wallner G, Erhart R, Amann R (1995) Flow cytometric analysis of activated sludge with rRNA-targeted probes. Appl Environ Microbiol 61:1859–1866
11. Ziglio G, Andreottola G, Barbesti S, Boschetti G, Bruni L, Foladori P, Villa R (2002) Assessment of activated sludge viability with flow cytometry. Water Res 36:460–468
12. Andreottola G, Foladori P, Gelmini A, Ziglio G (2002) Biomass active fraction evaluated by a direct method and respirometric techniques. Water Sci Technol 46:371–379
13. Nebe-Von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J Microbiol Methods 42:97–114
14. Veal DA, Deere D, Ferrari B, Piper J, Attfield PV (2000) Fluorescence staining and flow cytometry for monitoring microbial cells. J Immunol Methods 243:191–210
15. Vives-Rego J, Lebaron P, Nebe-Von-Caron G (2000) Current and future applications of flow cytometry in aquatic microbiology. FEMS Microbiol Rev 24:429–448
16. Joux F, Lebaron P (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect 2:1523–1535
17. Nielsen JL, De Muro MA, Nielsen PH (2003) Evaluation of the redox dye 5-cyano-2, 3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence in situ hybridization. Appl Environ Microbiol 69:641–643
18. Mortimer FC, Mason DJ, Gant VA (2000) Flow cytometric monitoring of antibiotic-induced injury in Escherichia coli using cell-impermeant fluorescent probes. A A C 44:676–681
19. Yamaguchi N, Sasada M, Yamanaka M, Nasu M (2003) Rapid detection of respiring *Escherichia coli* O157:H7 in apple juice, milk, and ground beef by flow cytometry. Cytometry A 54:27–35
20. Frolund B, Griebe T, Nielsen H (1995) Enzymatic activity in the activated sludge floc matrix. Appl Microbiol Biotechnol 43:755–761
21. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
22. HACH (1996–2000) HACH DR/4000 Spectrophotometer Handbook, Method 8000, HACH Company, USA
23. Falcioni T, Manti A, Boi P, Canonico B, Balsamo M, Papa S (2006) Comparison of disruption procedures for enumeration of activated sludge floc bacteria by flow cytometry. Cytometry B Clin Cytom 70:149–153
24. Valo A, Carrère H, Delgenès JP (2004) Thermal chemical and thermo-chemical pre-treatment of waste activated sludge for anaerobic digestion. J Chem Technol Biotechnol 19:1197–1203
25. Paul E, Camacho P, Lefebvre D, Ginestet P (2006) Organic matter release in low temperature thermal treatment of biological sludge for reduction of excess sludge production. Water Sci Technol 54:59–68