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Título da tese

Supervision of Transient Anaerobic Granular Sludge Process through Quantitative Image Analysis and Multivariate Statistical Techniques

Supervisão de processos anaeróbios com biomassa granular, em condições transientes, através de análise de imagem quantitativa e técnicas estatísticas multivariáveis

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Ano de conclusão 2008

Doutoramento em Engenharia Química e Biológica

Universidade do Minho, 31 de Julho de 2008

AGRADECIMENTOS

Finalmente... Não sei bem porquê mas esta é sempre a última parte a ser escrita nas teses. Chegado este momento significa que toda a parte *científica* está terminada, vista e revista, e só me falta expressar o agradecimento a todos que, de uma forma directa ou indirecta, contribuiram para que estes quatro anos fossem recheados de objectivos conquistados, tanto a nivel científico como pessoal.

Como não podia deixar de ser as minhas primeiras palavras de gratidão são para os meus orientadores científicos: o Doutor Eugénio Ferreira e a Doutora Madalena Alves. Quando, dois anos após ter terminado a licenciatura, decidi regressar à universidade e fazer o doutoramento, desde logo a primeira pessoa que pensei em contactar foi o Doutor Eugénio que já tinha orientado o meu projecto e estágio curricular. A sua imediata disponibilidade e entusiasmo na delineação das linhas mestres deste doutoramento, bem como as suas pontuais, mas precisas, sugestões ao longo dos últimos anos foram fundamentais para o sucesso não só do doutoramento mas do meu percurso académico. Após quase um ano de doutoramento, em que as coisas pareciam não querer evoluir, foi a Doutora Madalena Alves quem me recolocou no caminho certo e me fez avançar na pesquisa com toda a energia. Lembro-me especialmente de uma frase, algo parecido com: *"o sucesso faz-se com 90 % de transpiração e 10 % de inspiração"*. Tenho a certeza que os 90% de trabalho árduo estão de alguma forma condensados nesta tese. Só espero que os outros 10% também sejam visiveis e suficientes...! O seu constante incentivo e toda a discussão cientifica gerada, principalmente nas horas passadas a revêr os artigos, foram extremamente valiosas para a minha aprendizagem e o sucesso deste trabalho.

Penso que a vida de investigação tem um sentido completamente diferente depois de se conhecer a vida empresarial, com a visão mais realista(?) que o dia-a-dia na indústria nos oferece. Esta oportunidade foi-me concedida pela C.P.A., através do contacto com diversas indústrias aquando da minha passagem como consultor ambiental. Penso que, por tudo isso, merecem uma palavra de agradecimento, especialmente o Eng.º Mário Aguilar que me *"guiou"* nos primeiros dois anos após terminar a licenciatura em Engenharia Biológica.

À Olga e ao Pablo devo muitas das técnicas laboratoriais e de operação de reactores que adquiri durante estes quatro anos. Uma vez que nunca tinha feito investigação antes de começar o doutoramento, foi a sua experiência e ensinamentos que facilitaram imenso o trabalho que viria a ser realizado posteriormente.

Obviamente o sucesso de qualquer trabalho de investigação está directamente correlacionado (após quatro anos a ouvir estes termos é dificil dissociarmo-nos deles, mesmo numa conversa mais lúdica como esta...) com o grupo de trabalho onde se é inserido. Felizmente tive a sorte de trabalhar com grupos altamente capacitados. Portanto, aqui fica o meu agradecimento a todos os elementos do Laboratório de Biotecnologia Ambiental (LBA) e do BioProcess Systems Engineering group (BioPSEg) , que através de várias sugestões e incentivos tornaram este momento possivel. No entanto, duas pessoas merecem uma palavra de destaque especial: Ângela e Isabel, sem a vossa ajuda esta investigação teria sido impossivel. Operar e monitorizar reactores durante 24 horas por dia seria uma tarefa impossivel sem a vossa disponibilidade, muitas vezes para trabalhar até altas horas da madrugada.

Ana Julia, Ana Paulo, Andreia, Ângela, Catarina, Daniela, Diana, Isabel, Joana e Óscar. Vocês merecem um destaque especial, não só por toda a ajuda prestada ao longo destes quatro anos, mas principalmente por terem sido os maiores responsáveis por tornar este doutoramento, não só num trabalho mas também num divertimento. Depois de quatro anos de convivio (com alguns já um pouco mais) e muitos bons momentos, ja não os posso considerar apenas simples colegas de trabalho. São amigos que ficarão sempre marcados na minha vida.

No Departamento de Engenharia Biológica tive a sorte de encontrar um grupo de investigadores altamente vocacionados para a prática desportiva (um dos meus hobbies preferidos). À equipa DEB&Win e a todos os seus elementos (não menciono nomes para não cair no erro de me esquecer de algum) que durante quatro anos disputaram acesos jogos de futebol e voleibol, obrigado por fazerem jus ao lema *"mente sã em corpo são"*, e por todos os troféus conquistados em torneios como a Liga de Futsal NEEB e o Troféu Reitor de Futsal e Voleibol. Foi convosco que passei alguns dos melhores momentos "extra ciência" destes últimos anos.

Não posso deixar passar esta oportunidade sem expressar toda a minha admiração por uma pessoa que acompanha o meu caminho à 8! anos. Quando todas as minhas energias se esgotam é às tuas que recorro. Só o teu apoio e incentivo me permitiram alcançar mais esta meta. Obrigado Natália por tudo que vivemos juntos.

Sempre pensei que não ia passar uma página de agradecimentos, mas afinal...

A última palavra tem de ser para a minha familia. Aos meus irmãos que sempre me apoiaram em todas as decisões da minha vida. E aos meus pais pela invulgar, mas excepcional família que me deram. Esta é a melhor oportunidade para vos dizer que apesar de tudo que tiveram que passar para "criar" esta família, valeu a pena... sois o meu melhor modelo de perseverança e de história de vida!

The work presented in this thesis was financially supported by a research grant SFRH/BD/13317/2003 from the Fundação para a Ciência e a Tecnologia (FCT).

SUMMARY

Anaerobic technology is widely applied for wastewater treatment, while production of a renewable source of energy, the biogas, is achieved. In the last decades we assisted the spreading of high-rate anaerobic reactors based on granular sludge due to its high efficiency and stability. However, restocking granules to substitute those lost by excessive granular sludge breakdown and consequent washout is a common problem in these reactors. Since the granular sludge is an expensive product, its stability and maintenance is a sensitive issue that justify an appropriate attention and study. Some of the most common problems found in anaerobic processes are caused by a sudden increase in the feed flow rate and/or in the organic concentration, and the presence of toxic compounds in the feeding.

The research presented in this thesis was approached in two stages: first, organic load disturbances and toxic shock loads were applied to lab-scale Expanded Granular Sludge Bed (EGSB) reactors in order to simulate the effects caused by these transient situations; and, secondly, a chemometric technique entitled Principal Components Analysis (PCA) was performed in datasets gathering reactor performance, physiological, and morphological information, to visualize the importance of the proposed parameters to detect, on time, the transient situations simulated. Quantitative image analysis techniques were used to describe the micro and macro structural changes of anaerobic granular sludge during the transient periods tested.

Four organic loading disturbances of 18.5 $kg_{COD}\cdot m^{-3}\cdot d^{-1}$ (LD1 and LD2) and 50 $kg_{COD}\cdot m^{-3}\cdot d^{-1}$ (LD3 and LD4) were performed in lab-scale EGSB reactors fed with ethanol. In the organic shocks imposed by increasing the influent concentration (LD1, LD3 and LD4) it was clearly observed a decrease in aggregates size, since the percentage of aggregates projected area with equivalent diameter (D_{eq})≥1mm had a reduction between 22% and 45%. In general, it was observed the selective washout of filamentous forms associated to granules erosion/fragmentation and to a decrease in the specific acetoclastic activity. These phenomena induced the transitory deterioration of reactor performance in LD2, LD3 and LD4, but not in LD1. Evaluating reactor's performance, it was concluded that stability in the granules size distribution is of minor importance when compared to the capacity of filaments retention in the granular microbial structures. Extending the exposure time (LD4) promoted acetogenesis inhibition after 144h. The ratio filaments length per total aggregates area (LfA) could not be used as an early-warning indicator of washout because the increase in effluent volatile suspended solids (VSS) occurred immediately when the disturbances were applied.

A commercial detergent and a solvent were fed to lab-scale EGSB reactors to detect the effects of toxic compounds from cleaning stages. The inhibitory effects were dependent on surfactant concentration and exposure time. The COD removal efficiency remained unaffected with 150 $mg_{COD\text{-}detergent}\cdot L^{-1}$, but 80 h after starting exposure to 300 $mg_{COD\text{-}detergent}\cdot L^{-1}$ the COD removal efficiency decreased drastically from 75 to 17%. Relatively to the solvent shock load, in preliminary batch assays, 33 $mg_{solvent}\cdot L^{-1}$ caused 50% relative biomass activity loss. However, in the EGSB reactor, fed with 40 $mg_{solvent}\cdot L^{-1}$, the reactor performance seemed to be unaffected. The % of aggregates projected area with D_{eq}≥1mm decreased from 81 to 53 %, coupled with the settling velocity decrease, showing that the granules experienced a fragmentation phenomenon. The LfA parameter was an early-warning

indicator of biomass washout, in toxic shock loads, since it increased some days before the effluent VSS rise.

In the last decade, reports about monitoring and control of anaerobic wastewater treatment technology do not consider the integration of quantitative morphological indicators. The application of PCA to datasets obtained during transient states of high-rate anaerobic reactors determined a latent variable that encompasses a weighted sum of performance, physiological and morphological information. This new variable was highly sensitive to reactor efficiency deterioration, enclosing remarkable variation in the first hours of the disturbances. The high loadings raised by image analysis parameters revealed that morphological changes of solid phase (biomass) should be considered to monitor and control load disturbances and toxic events in high rate anaerobic (granular) sludge bed digesters.

SUMÁRIO

A tecnologia anaeróbia é amplamente aplicada no tratamento de águas residuais, permitindo, simultâneamente, obter uma fonte de energia renovável, o biogás. Nas últimas décadas assistimos ao alastrar de digestores anaeróbios de altas cargas, baseados em biomassa granular, devido à sua alta eficiência e estabilidade. Contudo, substituir os grânulos perdidos devido à sua fragmentação e consequente "washout", é um problema comum neste tipo de reactores. Devido ao elevado preço da biomassa granular, a sua estabilidade e manutenção é uma assunto sensivel que justifica o devido estudo. Alguns dos problemas mais comuns encontrados em processos anaeróbios de tratamento de águas residuais são causados por repentinos aumentos do caudal de alimentação e/ou da concentração orgânica, e pela presença de compostos tóxicos.

A investigação apresentada nesta tese foi abordada em duas etapas. Foram aplicadas a reactores laboratoriais de biomassa granular de leito expandido (EGSB) perturbações na carga orgânica e choques com tóxicos de forma a simular os seus efeitos em situações transientes. Posteriormente, uma técnica de quimiometria, denominada Análise de Componentes Principais (PCA), foi aplicada a bases de dados que reunem informação da performance do reactor, e da fisiologa e morfologia da biomassa, de forma a visualizar a importância dos parâmetros propostos para detectar, atempadamente, as diversas situações simuladas. Técnicas de análise de imagem quantitativa foram utilizadas com o objectivo de ilustrar as alterações na micro e macro estrutura da biomassa anaerobia granular durante os periodos transientes testados.

Foram realizadas quatro perturbações na carga orgânica de 18.5 $kg_{CQO}\cdot m^{-3}\cdot d^{-1}$ (LD1 e LD2) e 50 $kg_{CQO}\cdot m^{-3}\cdot d^{-1}$ (LD3 e LD4) em reactores laboratoriais EGSB, alimentados com etanol. Nos choques orgânicos originados pelo aumento da concentração orgânica no afluente (LD1, LD2 e LD3) foi observado um decréscimo no tamanho dos grânulos, uma vez que a % da área projectada de grânulos com diâmetro equivalente (D_{eq}) ≥ 1 mm sofreu uma redução entre 22 e 45%. No geral, foi observada a saida selectiva de filamentos associada à erosão/fragmentação dos grânulos e ao decréscimo da actividade especifica acetoclástica. Estes fenómenos induziram uma deterioração temporária na eficiência do reactor em LD2, LD3 e LD4, mas não em LD1. Avaliando a performance dos reactores, pode concluir-se que a estabilidade do tamanho dos grânulos é de menor importância quando comparada com a capacidade de retenção dos filamentos na estrutura microbiana dos grânulos. O prolongamento do tempo de exposição (LD4) promoveu a inibição da acetogénese após 144h de choque. A razão entre o comprimento dos filamentos e a área total dos agregados (LfA) nao pôde ser usado como um indicador de alerta rápido de "washout" porque o aumento dos sólidos suspensos voláteis (SSV) no efluente ocorreu imediatamente após aplicação da perturbação.

Com o objectivo de detectar os efeitos causados por compostos tóxicos provenientes de etapas de limpeza foram alimentados a reactores EGSB, um detergente e um solvente. Os efeitos inibitórios dependeram da concentração de surfactante e do tempo de exposição. A eficiência de remoção de CQO não foi afectada com 150 $mg_{CQO\text{-}detergente}\cdot L^{-1}$, mas 80 h após o incio da exposição a 300 $mg_{CQO\text{-}detergente}\cdot L^{-1}$ a eficiência decresceu drasticamente de 75 para 17%. Relativamente ao choque com solvente, em testes *batch* preliminares, foi observado que 33 $mg_{solvente}\cdot L^{-1}$ causaram um decréscimo de 50% na actividade da biomassa. Contudo, a performance do reactor, alimentado com 40 $mg_{solvente}\cdot L^{-1}$, não foi afectada. A % da área projectada dos agregados com D_{eq} ≥ 1 mm decresceu de 81 para 53 %, simultâneamente

com o decréscimo da velocidade de sedimentação, demonstrando que os grânulos sofreram fragmentação. O parâmetro LfA foi um indicador de alerta rápido do "washout" da biomassa, uma vez que aumentou alguns dias antes do aumento dos SSV no efluente.

Na última década, os relatórios existentes sobre monitorização e controlo de processos de digestão anaeróbia não têm em consideração indicadores quantitativos da morfologia da biomassa. A aplicação de PCA a bases de dados, obtidas durante estados transientes em reactores anaeróbios de alta carga, determinou uma variável latente que engloba uma soma ponderada da informação relativa à performance do reactor, e da fisiologia e morfologia da biomassa. Esta nova variável foi altamente sensivel à deterioração da eficiência do reactor, demostrando uma assinalável variação nas primeiras horas de perturbação. Os pesos elevados, associados aos parâmetros de análise de imagem, revelam que as alterações morfológicas da fase sólida (biomassa) devem ser consideradas na monitorização e controlo de reactores anaeróbios de alta carga, operando com biomassa granular, durante perturbações na carga orgânica e presença de tóxicos.

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LIST OF SYMBOLS AND ABBREVIATIONS

ABBREVIATIONS

AD	Anaerobic Wastewater Treatment Technology
CCD	Charge Coupled Device
CLSM	Confocal Laser Scanning Microscopy
CMOS	Complementary Metal Oxide Silicon
CMYK	Colour space based in the Cyan, Magenta, Yellow and Black channels
COD	Chemical Oxygen Demand
DGGE	Denaturant Gradient Gel Electrophoresis
ECP	Exocellular Polymers
EMAS	Eco-Management and Audit Scheme
FISH	Fluorescence In Situ Hybridisation
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
HSI	Colour space based in Hue, Saturation and Intensity
ISO 14000	International standard for an environmental management system
JPEG	Joint Photographers Expert Group
LAS	Linear Alkylbenzene Sulfonate
LCFA	Long Chain Fatty Acid
LD	Load Disturbance
OHPA	Obligate Hydrogen Producing Acetogens
OLR	Organic Loading Rate
OP	Operation Period
PHB	Poly-β-hydroxybutyrate
RGB	Colour space based in the Red, Green and Blue channels
SAA	Specific Acetoclastic Activity
SEM	Scanning Electron Microscopy
SHMA	Specific Hydrogenotrophic Methanogenic Activity
SL	Shock Load
SMA	Specific Methanogenic Activity
SRT	Solids Retention Time
STP	Standard Temperature and Pressure Conditions
SVI	Sludge Volume Index
TEM	Transmission Electron Microscopy
TIFF	Tagged Image File Format

TOC	Total Organic Carbon
TPE-LSM	Two Proton Excitation Laser Scanning Microscopy
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

MULTIVARIATE STATISTICAL ANALYSIS

$\boldsymbol{\lambda}$	Eigenvalue
$\boldsymbol{C}$	Covariance Matrix
cov	Covariance
CV	Cross Validation
$\boldsymbol{Dim_x}$	x^{th} Dimension of the Matrix
$\boldsymbol{E}$	Matrix of Residuals
$\boldsymbol{e}$	Eigenvector
$\boldsymbol{I}$	Identity Matrix
$\boldsymbol{P}$	Matrix of Loadings
$\boldsymbol{i}$	Sample
$\boldsymbol{j}$	Variable
$\boldsymbol{p_a}$	Loadings Vector
PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial Least Squares Analysis
PRESS	Prediction Error Sum of Squares
$\boldsymbol{S_{i,\mathrm{PC1}}}$	Score of sample i on Principal Component 1
$\boldsymbol{s_j}$	Standard Deviation of Variable j
SS	Residual Sum of Squares
T	Matrix of Scores
$\boldsymbol{t_a}$	Score Vector
$\boldsymbol{w_{j,\mathrm{PC1}}}$	Weight/Loading of Variable j on Principal Component 1
$\boldsymbol{X}$	Data Matrix
$\boldsymbol{\overline{X}}$	Matrix of Variables Averages
$\boldsymbol{x_{ij}}$	Variable j in the sample i
$\boldsymbol{\overline{x}_j}$	Average of Variable j
$\boldsymbol{z_{ij}}$	Autoscaled variable of x_{ij}

REACTORS

AAO	AAO - Integrated Anaerobic - Anoxic - Aerobic System
AF	Anaerobic Filter
AFBR	Anaerobic Fluidized Bed Reactor
AOND	Integrated Anaerobic - Aerobic - Nitrification - Denitrification System

CSTR	Continuous Stirred Tank Reactor
EGSB	Expanded Granular Sludge Bed Reactor
HYBR	System with sludge bed at the bottom section and a filter in top (UASB + AF)
IC®	Internal Circulation Reactor
SBR	Anaerobic Sequencing Batch Reactor
UASB	Upflow Anaerobic Sludge Blanket Reactor
UFBR	Up-flow Anaerobic Fixed Bed Reactor

SYMBOLS

%Area	Total aggregates projected area distribution by equivalent diameter ranges
%CH_4	Methane Content in the gas phase
%CO_2	Carbon Dioxide Content in the gas phase
%Nb	Total number of aggregates distribution by equivalent diameter ranges
$\Delta G^{0,}$	Gibbs free energy under standard conditions
[CO]	Carbon Monoxide concentration in the gas phase
[H_2]	Hydrogen Concentration in the gas phase
[N-NH_4^+]	Nitrogen Concentration in the influent
<0.01	Percentage of Aggregates Projected Area with D_{eq} < 0.1 mm
>0.1	Percentage of Aggregates Projected Area within the range 0.1 ≤ D_{eq}(mm) < 1
>1	Percentage of Aggregates Projected Area with Equivalent Diameter ≥ 1 mm
A	Aggregates area
A_i	Area of aggregate i
Alk	Alkalinity
AR	Aspect Ratio
A_{spec}	Specific Area of Aggregates
$A_{spec<0.2mm}$	Specific Area of Aggregates with Equivalent Diameter < 0.2 mm
$A_{spec≥0.2mm}$	Specific Area of Aggregates with Equivalent Diameter ≥ 0.2 mm
A_T	Total Area of Aggregates
BF	Buffer Flow rate
BPF	Bypass Flow rate
CF	Compactness Factor
COD/N	Chemical Oxygen Demand to Nitrogen ratio in the influent
Conv	Convexity
d	Aggregates diameter
D_{eq}	Equivalent Diameter
Ecc	Eccentricity
EF	Elongation factor
Eff	COD Removal Efficiency

$\mathbf{e_{fil}}$	Filamentous fraction
$\mathbf{e_{flocs}}$	Flocs fraction
F	Feed flow rate
$\mathbf{F_{cal}}$	Calibration Factor (µm·pixel^{-1})
FD	Fractal Dimensions
Ff	Form Factor
fNb	Filaments Number
GF	Gas Flow rate
HCF	Heywood Circularity Factor
IA/TA	Intermediate to Total Alkalinity ratio
L	Filament Length
LfA	Total Filament Length to Total Aggregates Area Ratio
LFi	Filaments Length by Image
$\mathbf{L_{spec}}$	Specific Total Filament Length
MF	Methane Flow rate
N	Number of pixels of an object
Nb	Number of aggregates
P	Perimeter
RF	Recycle Flow rate
Rg	Reduced Gyration Radius
Ro	Roundness
S	Reactor Surface Area
Sol	Solidity
T	Temperature
TA	Total Aggregates Area
TL	Total Filaments Length
TL/TA	Ratio between total filaments length and total aggregates area
TL/VSS	Total Filament Length per Volatile Suspended Solids
V	Reactor Volume
$\mathbf{V_{field}}$	Volume of the field of view/image
VSS/TA	Volatile Suspended Solids per Total Aggregates Area
W	Width.
v	Up-flow Velocity
v_{sed}	Settling Velocity

Context, Aim and Outline of the Thesis

ABSTRACT

The fundamentals on anaerobic wastewater treatment technology as the core process under study, and, on image analysis and multivariate statistical techniques as the main tools to monitor and/or control it, are briefly presented. The perspectives and motivation of this research is discussed. Finally, the research aim and outline of the thesis are specified.

1.1 ANAEROBIC DIGESTION CONCEPTS

Since the last century we assist to an exponential increase of population and industrialization that guide us to an uncontrolled development and consequent contamination of air, soil and water. The urgent need for environmental protection, with emphasis for water quality, forced the investment in research and development of new technologies that could overcome, or at least minimize, these problems. In the last decades of the twentieth century the anaerobic wastewater treatment technology (AD) emerged to beat against the well established activated sludge process.

Anaerobic Digestion is the *biological degradation, by a complex microbial ecosystem, of organic and occasionally inorganic substrates in the absence of oxygen*. It occurs naturally in ponds sediments, the deep sea, marshes, and also in the intestine of several animals (Hanson and Hanson, 1996). During the process, organic material is converted to mainly methane, carbon dioxide and biomass (Figure 1.1) through four main steps:

- **Hydrolysis** is an extracellular step mediated by hydrolytic enzymes. During this first step polymers that cannot be directly utilised by the anaerobic consortium are converted to their basic building units (monomers).
- In the **Acidogenesis (or Fermentation)** step, soluble substrates such as amino acids, sugars, and glycerol, which can be degraded largely without an external electron acceptor, are converted mainly to organic acids and alcohols.

- Subsequently, through syntrophic relationships, the degradation of fermentation products to acetate using hydrogen ions or bicarbonate as an external electron acceptor occurs, in a process known as **Syntrophic Acetogenesis**. This process is coupled with hydrogen or formate utilising methanogenesis, which maintains a low hydrogen or formate concentration. In many studies (including this one), the electron acceptor product (hydrogen or formate) is referred to as hydrogen as the hydrogen/bicarbonate couple is thermodynamically and stoichiometrically similar to formate (Schink, 1997).
- Finally, the **Methanogenesis** step consists in the production of methane from the cleavage of acetate (acetoclastic methanogenesis) and uptake of hydrogen (hydrogenotrophic methanogenesis) via highly specialised organisms.

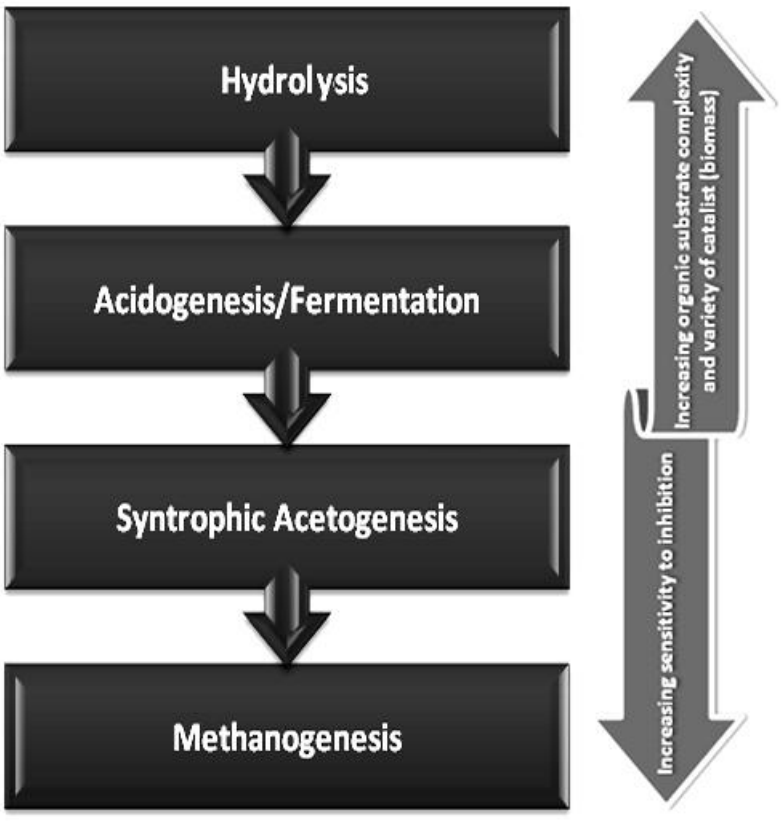

Figure 1.1 – Schematic representation of the four main steps for anaerobic degradation of complex compounds.

The small amount of energy available in the methanogenic conversion forces the microorganisms involved into a very efficient cooperation. As can be seen in the conversion of hexose to methane and carbon dioxide, which releases only 15% of the energy that would be available in aerobic degradation (Schink, 1997):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad (\Delta G^{0'} = -2\,870\ kJ \cdot mol^{-1}) \qquad (1.1)$$

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \qquad (\Delta G^{0'} = -390\ kJ \cdot mol^{-1}) \qquad (1.2)$$

The mutual dependence of the anaerobic consortium with respect to energy limitation, may imply that none of the partners involved in the process can operate without the other and that together they exhibit a metabolic activity that neither one could accomplish on its own.

Although the first and most important objective of AD is the wastewater treatment itself, it can also be considered as a profit-possible technology. With the energy prices repeatedly rising and the overall concern on global warming, AD can be seen as a renewable energy source since the process produces a methane and carbon dioxide rich biogas, which is suitable for energy production helping replace fossil fuels. According to van Lier (2007) an agro-industrial wastewater (25 tonsCOD/day) can be converted in 7000 m^3_{CH4}/day (assuming 80% CH_4 recovery), with an energy equivalent of about 250 GJ/day. Working with a modern combined heat power gas engine, reaching 40% efficiency, a useful 1.2 MW electric power output can be achieved. Assuming that full aerobic treatment would require ± 1 kWh/$kg_{COD\text{-}removed}$, or 1 MW installed electric power in the above case, the total energy benefit of using anaerobic wastewater treatment over the activated sludge process is 2.2 MW. At an energy price of 0.1 €/kWh this equals about 5000 €/day.

Emphasis should be given to extra possibilities, such as, the carbon credits that can be obtained by generating renewable energy, and the excess biomass that can be sold as inocula for new reactors start-up. All these factors represent market opportunities, and act as an incentive, mainly to developing countries start treating

the wastewater using anaerobic technology, and thus achieving a sustainable development.

Although all the advantages associated with AD, due to its highly specialized character, the microorganisms involved in the process are very sensitive to disturbances of the normal operational conditions (Steyer et al., 1999). Effectively, sudden variations in flow or substrate concentrations in the influent can lead the process to overload or underload states. Another frequent problem in AD is the contamination by toxic compounds. These situations might cause destabilisation of the process resulting in efficiency falls, biomass disruption and washout. Due to the slow start-up of anaerobic wastewater treatment processes this problems can be transformed in several weeks to several months necessary for the reactor to recover if the appropriate measures/actions are not taken in time.

1.2 IMAGE ANALYSIS CONCEPTS

The technological evolution always opened new opportunities and presented different approaches that changed our routines, learnt during time. This evolution allows for difficult tasks to become more resourceful and effortless. Particularly in the last years we assist to the rise of digital technology, with huge developments, both in computers and digital cameras. Consequently, in this *digital era*, it was just a step to see applications of these technologies in a wide range of fields. In biotechnology, the tedious, subjective, and time consuming techniques used in traditional classification and quantification of microorganisms are being replaced by automated and semi-automated quantitative image analysis techniques (Ginoris et al., 2007a; Coskuner et al., 2005) based in digital technology.

In fact, the human vision system is superb, but qualitative. Computers can do better than humans at extracting quantitative information and can reduce the tedious aspects of image interpretation. Image analysis can be seen as *the simple extraction of information from pictures* (Glasbey and Horgan, 1995). These pictures are the sum of a vast number of tiny points, called pixels. Together they give us a qualitative image, however after processing and analysing, it allow us the extraction of indispensable quantitative information.

The image analysis method, in a wide sense, refers not only to the strict image analysis process but also to the previous processes of image capture and processing (Dougherty, 1994). The overall process can be summarised in three main steps:

- After sampling and preparation, an image is **captur**ed with the help of an optical, fluorescence, confocal laser scanning microscope (Lopez et al., 2005) or electron microscope. Then, the images are obtained by means of digital cameras and saved on magnetic or optical data carriers with the use of specialized software.
- Image **process**ing consists in a set of operations performed to transform an image in order to enable the measurement of the observed objects. Another task of this step is to improve the quality of the images by reducing noises, enhancing objects and detecting their edges. Basic tools are point operations and filtration in the frequency and space domain by means of linear and non-linear filters. The processed image is then subjected to segmentation and as a result a binary image is obtained.
- Finally, morphological parameters are **measure**d. Usually, the objective of image analysis concerns (Liwarska-Bizukojc, 2005): morphology, *i.e.* the size and shape of objects; composition of objects, *i.e.* exploration of their internal structure, for example distribution of microbes; identification of microbial species; and, spatial arrangement of microorganisms.

Quantitative image analysis techniques may overcome some gaps bringing new opportunities in monitoring/control of wastewater treatment processes. During the last years little importance has been credited to morphological indicators because of the difficulties found in acquire accurate and quantitative measurements. The use of image analysis has been widely studied in activated sludge processes, either in the identification of protozoa and metazoa populations (Amaral et al., 2004), or in the correlation with settling characteristics of the biomass, with prominence to sludge volume index (SVI) (Amaral and Ferreira, 2005). However, the number of programmes specially developed and applied in anaerobic wastewater treatment processes is smaller. Nevertheless, image analysis techniques were successfully applied in the detection of critical events in anaerobic processes, such as granulation time (Araya-Kroff et al., 2004) and biomass activity recovery (Abreu et al., 2007).

1.3 CHEMOMETRICS CONCEPTS

Chemometrics, occasionally entitled environmetrics when applied in the field of environmental science has been extensively applied in the last decades. It can be defined as *the chemical discipline that uses mathematical, statistical, and other methods employing formal logic to: design or select optimal measurement procedures and experiments; and, provide maximum relevant information by analysing data* (Massart et al., 1988).

Some decades ago, the amount of instrumentation in environmental processes was minimal. However, with the rapid development of instrumental methods the amount of diverse data generated in an environmental process monitoring and/or control is increasingly drastically (Bourgeois et al., 2001; Schügerl, 2001; Spanjers and van Lier, 2006). This advance, guide analysts and researchers to gathering

further more multivariate data in several working fields, such as wastewater treatment. Concurrently, with computer science and technology developments apply computers and advanced statistical and mathematical methods to analyse this data became easier.

One problem with multivariate data is that its absolute volume may create difficulties in patterns and relationships recognition. Therefore the aim of many methods of multivariate analysis is data reduction. Quite frequently there is some correlation between the variables, and so some of the information is redundant. Principal Components Analysis is a technique for reducing the amount of data when there is correlation present. It can be used to (Miller and Miller, 2000):

- Identification of groups of inter-related variables;
- Reduction of the number of variables;
- As a method of transforming data. Transformation of data through rewriting the data with properties that the original did not have. The data may be efficiently simplified prior to a classification while removing artefacts such as multicollinearity.

A few multivariate statistical analysis have been used together with image analysis techniques to pattern recognition, such as discriminant analysis (Amaral et al., 2004), and, neural networks and decision trees (Ginoris et al., 2007b). The relationships between morphological parameters and biomass properties in aerobic wastewater treatment processes were also assessed by partial least squares regression (Amaral *et al.*, 2005) and principal components analysis (Jenné et al., 2006).

1.4 PERSPECTIVE AND MOTIVATION

As deeply the world walks to economical and social development the amounts of waste and wastewater produced increases mutually. These emissions can constitute a threat to water quality and public health when released directly, without treatment, to the environment. Consequently, in the last years has being observed a growing environmental concern in society, compelling the industries and politics to take measures to overcome these problems. In this frame, wastewater treatment is of crucial relevance to achieve the sustainable development incorporated in the Rio de Janeiro's declaration (UNEP, 1992). This declaration followed by the Kyoto Protocol (UNEP, 1998) where is set that states should "*Implement and/or further elaborate policies and measures in accordance with its national circumstances, such as: // research on, and promotion, development and increased use of, new and renewable forms of energy //*" manifest the political positions in this field.

Concomitantly, industries are being pressured by politics and society to look at the environment as a market opportunity and not only as pointless costs. On one hand the application of the so-called *green-policies* is forcing the municipalities and industries to minimize the emissions and/or its negative effects. On the other hand, the percentage of society that favours *green industries and/or products* (e.g. environmentally certified by the ISO 14000 or EMAS) is growing. Together, they are gradually making industrials attitude changes by looking at waste and wastewater treatment as a possible investment to achieve financial and/or competition benefits. Merging wastewater treatment with renewable energy production is directing forces towards the most pressing challenges facing society: water and energy sustainability.

For many years activated sludge processes were almost the only technology for wastewater treatment. However, in the scope described previously, anaerobic technology emerged as a well established technology either as a mean to reduce

emissions (e.g. less production of biomass or untreated wastewater), and as a mean to produce renewable energy (e.g. biogas).

In Table 1.1 are summarised the main advantages and disadvantages of anaerobic wastewater treatment technology (Gravilescu, 2002; Speece, 1996; van Lier, 2007).

Table 1.1 – Advantages and disadvantages of anaerobic wastewater treatment technology.

ADVANTAGES	DISADVANTAGES
No aeration costs	Lack of an established nutrient removal capability
Reduction of excess sludge production up to 90%, and better stabilisation of biomass (*i.e.*, biomass generally does not decay further)	The incapability of treatment to secondary standard and poor performance on low strength wastewater
Production of a by-product (biogas) which can be used for heating and power generation	Usually, high investment costs
Lower footprint and higher space-time loading	Few toxic compounds allowed
Better process stability (when system is matched to loads)	Need post-treatment, because effluent cannot achieve most discharge standards
High applicable Organic Loading Rates (up to 40 $kg_{COD} \cdot m^{-3} \cdot d^{-1}$)	Lack of models and control systems
Smaller reactors required (90% reduction in space requirement when using expanded sludge bed systems)	
No or very little use of chemicals	
Anaerobic sludge can be stored unfed and the excess sludge has a market value	

The breakthrough for industrial anaerobic wastewater treatment applications occurred in the mid-seventies/eighties of the last century, following the development of anaerobic sludge bed technology at lab and pilot scale (Lettinga et al., 1980).

In January 2007 there were 2266 registered full scale installations in operation. Plus, at least about 500 *homemade* reactors constructed by local companies or by industries themselves (van Lier, 2007), and excluding thousands of anaerobic lagoons installed in Latin America, China and India (Frankin, 2001). However, for the analysis of trends it is assumed that these registered installations represent a good overview of *engineered systems* applied for anaerobic industrial wastewater treatment.

The development of high-rate reactors was the key feature that allowed for a great increase in the use of anaerobic technology for the treatment of a growing variety of industrial wastewaters (Lettinga, 1995). Uncoupling the hydraulic retention time from the solids retention time allows the application of high organic loading rates, making possible the use of compact and economical wastewater treatment plants. The first reactor using this concept was when, in 1968, James C. Young developed the anaerobic filter (Young and McCarty, 1969) that used inert carriers to immobilise the biomass. However, the boost of anaerobic technology was achieved with the development of the Upflow Anaerobic Sludge Blanket (UASB) reactor by Gatze Lettinga in the early seventies of the twentieth century (Lettinga et al., 1980). In this technology, under certain conditions, microorganisms can self-immobilise to form dense granular aggregates (Liu et al., 2003). According to Frankin (2001), UASB reactors represented 66% of the total anaerobic installations in the period 1990-1996. Also, in the last 5 years (2002–2007) the average of granular technology based installations (UASB, EGSB and IC) increased from 73 % (1981 to 2001) to 89 % (Figure 1.2).

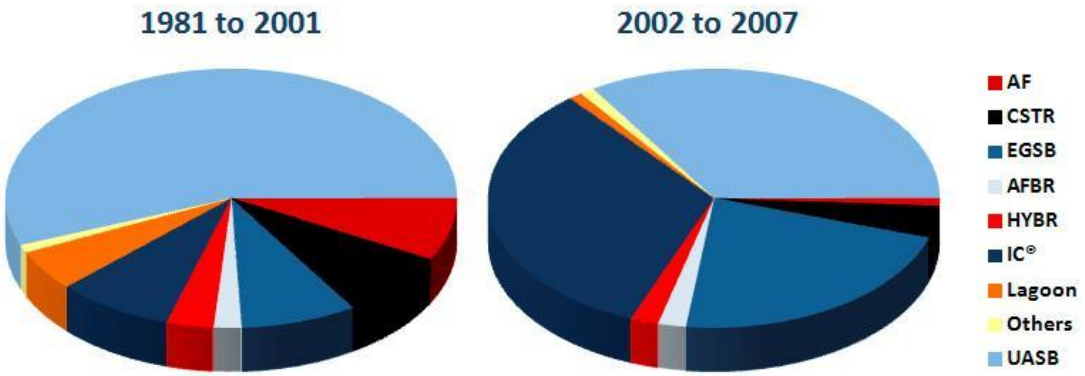

Figure 1.2 – Anaerobic digestion full-scale industrial installations (adapted from van Lier, 2007).

Analysing the last 5 years (2002-2007), it is apparent that the traditional UASB system is gradually being replaced by the *expanded* systems. Effectively the installations with this technology (EGSB, IC and AFBR) increased from 19 to 57 % (Figure 1.2), and the UASB system decreased from 56 to 34 % in the same period. The Expanded Granular Sludge Bed (EGSB), thanks to its small footprint, high liquid and gas superficial velocities (Kato et al., 1994), can operate with higher organic loading rate (OLR) than UASB reactor, being therefore economically more attractive.

It is important to enhance that granular morphology plays also an important role by protecting bacteria against adverse environmental conditions. Methanogenic acetoclastic bacteria that are known to be the most sensitive trophic group (Speece, 1996) in the anaerobic consortium are located mainly in the core of a stratified granule (Ennik-Maarsen et al., 1998; Schnurer et al., 1999; Liu et al., 2002a). This fact allows them to be more protected against operational problems (overloads, pH oscillations, etc.) or presence of toxics compounds (Liu et al., 2002b).

AD is a mature wastewater treatment technology, with worldwide application. The highest percentages of installations are implemented in the agro-food (Driessen and Yspeert, 1999), and in the breweries and beverage industries (Connaughton et al., 2006) (Figure 1.3). In the last years the anaerobic technology is spreading by other

industries, with emphasis in distilleries (Moletta, 2005), pulp and paper (Thompson et al., 2001), and chemical (Razo-Flores et al., 2006) industries.

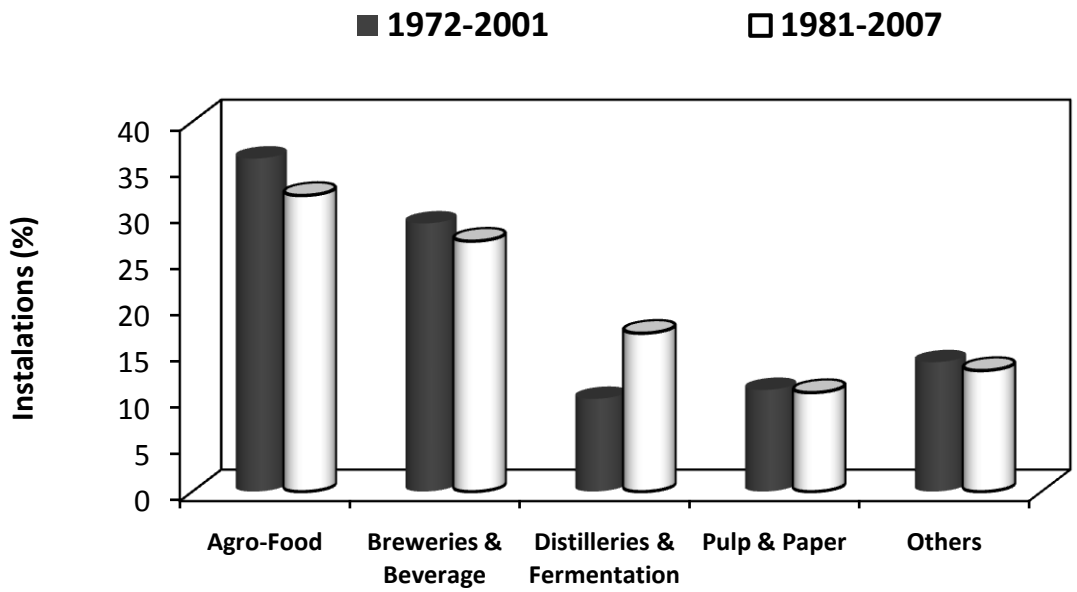

Figure 1.3 – Application of anaerobic technology in industrial wastewater (adapted from: ■ Frankin (2001), and □ van Lier (2007)).

1.5 RESEARCH AIM

The research work presented in this thesis aimed at studying the effects caused by organic loading disturbances and toxic shock events in high rate anaerobic reactors. Focus was targeted in quantifying morphological and physiological changes in anaerobic granular sludge through quantitative image analysis techniques.

One previous key aspect in monitoring and control of wastewater treatment plants is to define the most important and appropriate indicators to achieve an early detection of operational problems. With that purpose the chemometric technique

Principal Component Analysis was used in order to weight the several indictors/parameters monitored, with emphasis to the importance that the morphological indicators might have in detect the different disturbances. Enlighten the relationships between morphological, physiological and operating parameters, and, detect outliers, groups and/or trends in samples was also assessed.

1.6 OUTLINE OF THIS THESIS

The subject of this thesis and the basic concepts of the process studied and techniques applied in the framework of wastewater treatment and energetic valorisation were introduced in this chapter.

Later, the thesis is divided in 4 key sections (Figure 1.4). The first one refers to the *Introduction* (Chapter 2), followed by the *Methodology* (Chapter 3), the *Experimental Work* performed (Chapters 4, 5, 6 and 7), and the *General Conclusions* (Chapter 8). In every chapter of the third section a brief introduction, the experimental section, results and discussion, and conclusions for each specific subject are given.

A general overview on the current knowledge about anaerobic wastewater treatment technology, quantitative image analysis and multivariate statistical techniques is further presented in ***Chapter 2***. Several references are given to the reader who wants to study deeply each topic and/or the subject is just briefly discussed in this thesis.

The material and methods used during the research are explained in ***Chapter 3***. The Expanded Granular Sludge Bed Reactor used to perform the experiments is presented, as well as the parameters utilized to monitor its performance. Highlight is given to quantitative image analysis technique and specific methanogenic activity

assays. The Principal Components Analysis basics and respective mathematical background are also explained.

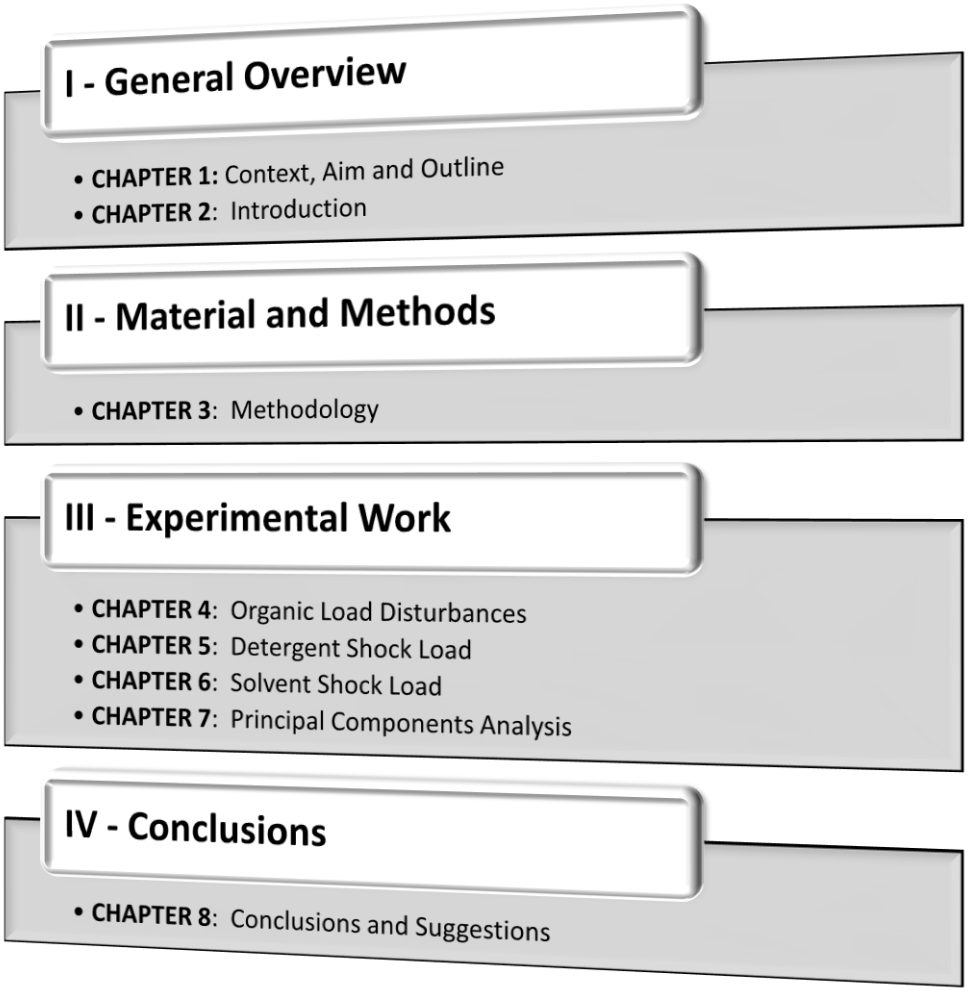

Figure 1.4 – Thesis Outline.

Organic loading disturbances are very recurrent in wastewater treatment processes in general, and in anaerobic processes in particular. A sudden increase in flow or concentration can initiate an overload of the system. ***Chapter 4*** presents the results and conclusions of four load disturbances in EGSB reactors. Emphasis was given in the effects caused by hydraulic retention time, substrate concentration, and exposure time.

Toxic events caused by undegradable/inhibitory compounds are another common problem found in anaerobic wastewater treatment technology. Detergents (or surfactants) and solvents are common products that can easily enter to a wastewater treatment system, either industrial or municipal, and deteriorate the reactors performance. Cleaning stages for example can generate high quantities of these products. Physiological and morphological effects of detergent shock loads in anaerobic granular sludge are presented in ***Chapter 5***. A commercial detergent, used in industry as cleaning agent was tested. In ***Chapter 6*** a solvent vastly used as cleaning agent in industry was feed to an EGSB reactor to evaluate the morphological effects caused in the granular sludge.

Chapter 7 describes the application of a multivariate statistical technique to highlight groups, trends, and outliers of the samples acquired during the procedures described previously in chapters 4 to 6. Because environmental data contains so much information, Principal Components Analysis was used to extract the latent information from these data. The benefit of use morphological indicators of the solid phase stability during load disturbances and toxic events was checked.

Finally, ***Chapter 8*** contains the concluding remarks and perspectives for further research inside this topic.

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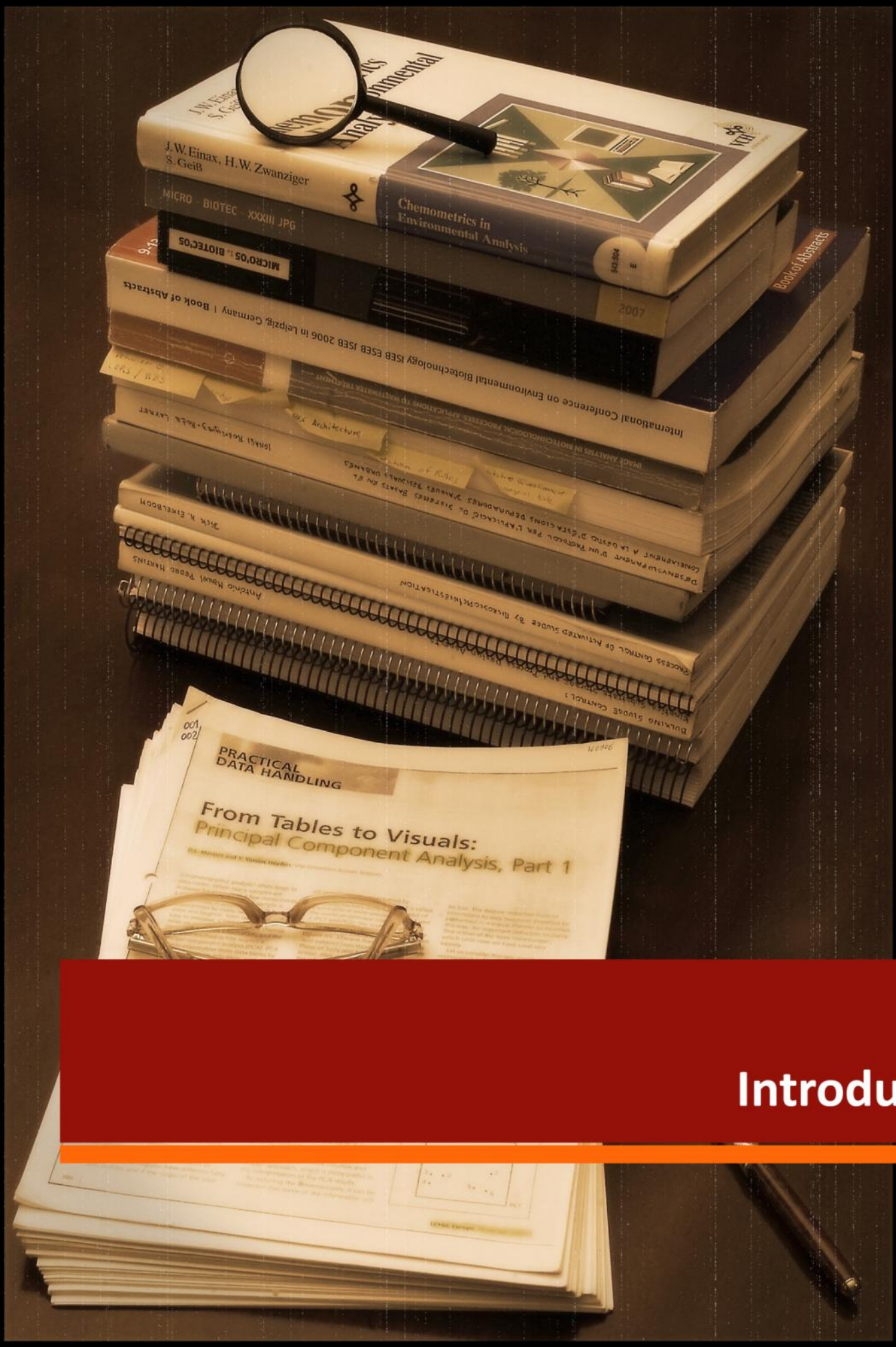

Introduction

ABSTRACT

A literature review of the main themes under study is presented. The anaerobic technology spot in the problematic of wastewater treatment including a brief description of the biochemical and the microbial fundamentals is given. The process under study is based on granular sludge, and so the main theories and advantages of these self-immobilized consortia of bacteria are discussed. The main compounds that can be toxic or inhibitory to anaerobic wastewater treatment technology are presented as well as the review of the main parameters and systems to monitor and control the process. As these systems continue to ignore the solid phase (biomass) monitoring during process disturbances, in a second part is described a quantitative method to evaluate the morphology of biomass. Review of image analysis parameters used in monitoring of wastewater treatment processes, both aerobic and anaerobic, is given. Finally, the utility of chemometric--based techniques, such as principal components analysis, in process supervision is explained.

2.1 THE PROBLEMATIC OF WASTEWATER TREATMENT

One of the hottest topics nowadays is the sustainable management of our environment. The survival of the human species and our quality of life depend upon our ability to manage the Earth's natural resources from local to global scales. This requires an assessment of the extension of these resources, including an understanding of their variation in time and space and of what causes these variations. The excessive concentration of population at specific locations and the vast industrialization are responsible for a non-sustainable exploitation of human resources and the equilibrium breaking of different natural ecosystems. It has resulted in environmental pollution, which affects negatively the quality of air, soil, and water, and consequently all life forms. The increasing degradation of the environment has forced the society to consider changes in human behaviour for ensuring the essential conditions for the life in Earth. This consideration has encouraged research and a great effort has been placed on preventing and correcting environmental degradation. In this sense, water and wastewater treatment has become one of the salient environmental issues. Wastewater treatment is fundamental to keep the water natural resources (rivers, lakes and seas) in as high quality as possible. Not only for this environmental reason, but also due to the more and more restrictive social regulations, the correct management of wastewater treatment facilities has become very important during the last decades.

2.2 THE ROLE OF ANAEROBIC TECHNOLOGY IN WASTEWATER TREATMENT

New renewable energy technologies constitute nowadays a hope for diversity and security of future energy supply. Therefore, promotion of energy from renewable sources is currently a priority for economical and social reasons, and for environmental protection. The use of anaerobic technology allows the conversion of the organic matter present in wastewater into biogas, a renewable energy source, while at the same time, and most important, wastewater treatment is accomplished.

Anaerobic technology has been widely used for wastewater treatment and methane production. In recent years, high rate technologies have been developed offering interesting and sustainable solutions for the energetic valorisation of wastewaters (Rajeshwari et al., 2000; Gijzen, 2002).

2.2.1 Brief History

Anaerobic Digestion can be considered as one of the oldest technologies for stabilising waste and wastewater. It has been applied since the end of the 19th century, mainly for the treatment of household wastewater in septic tanks, treatment of slurries in digesters and for the treatment of sewage sludge in municipal treatment plants (van Lier et al., 2001).

The first recognition that anaerobic biological processes result in the conversion of organic matter to methane is attributed to Volta, who in 1776 showed that *combustible air* was formed from sediments in lakes, ponds, and streams. In 1856 Reiset found methane being liberated from decomposing manure piles and proposed this process to be studied to help explain the decomposition of organic material in general (McCarty, 2001).

The conception of the first anaerobic digester remains unclear. According to McCarty (2001) it was built in France around 1860 and was defined as "*the most simple, the most beautiful, and perhaps, the grandest of modern inventions,*" and "*a complete solution of the problem which for centuries had been an insolent menace hurled in the face of all humanity*". The anaerobic technology then moved to Exeter–England in 1895, when biogas was recovered from a sewage system and used to fuel street lamps. In 1907, in Germany, a patent was issued for the Imhoff tank, an early form of digester.

Through scientific research anaerobic digestion gained academic recognition in the thirties of the twentieth century (Buswell and Hatfield, 1938). This research led to the discovery of anaerobic bacteria, the microorganisms that facilitate the process. In 1947 methanogenic bacteria (*Methanosarcina barkeri* and *Methanobacterium formicic*) were isolated in pure cultures for the first time (Zehnder et al., 1982). Further research was carried out to investigate the conditions under which methanogenic bacteria were able to grow and reproduce.

Since the late seventies, anaerobic wastewater treatment technology has experienced an outstanding growth in research and full-scale application, particularly for the treatment of industrial effluents (Totzke, 1999) and to a lesser extent of municipal wastewater, with prominence in tropical countries (Hulshoff Pol et al., 1997).

2.2.2 Biochemical and Microbial Fundamentals

The conversion of complex organic matter to methane and carbon dioxide proceeds through a series of parallel and sequential steps in which several groups of microorganisms are involved (Figure 2.1). The degree of mutual dependence among these different bacterial types varies considerably. Whereas the later members of the chain depend on the earlier ones for their substrates, they may also exert a

significant influence on the earlier members by removing metabolic (perhaps inhibitor) products (Schink, 1997). The course of this process is largely constrained by the electron acceptors present in the environment. In methanogenic environments, organic matter is decomposed in the absence of inorganic electron acceptors and, consequently, bicarbonate (carbon dioxide) and protons act as terminal electron acceptors (Stams et al., 2006).

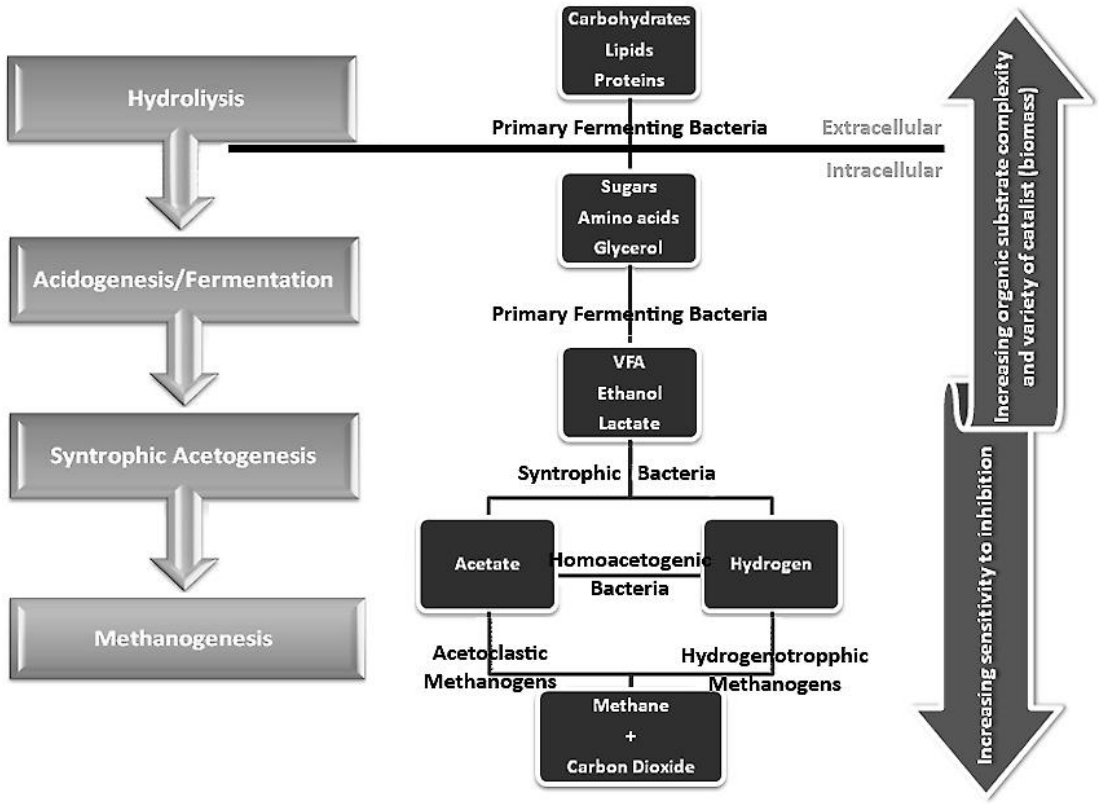

Figure 2.1 – Basic digestion pathways of anaerobic wastewater treatment technology. Main substrates and electron flow through the various trophic groups of microorganisms involved in methanogenic degradation of complex organic matter.

The digestion process (Figure 2.1) begins with enzymatic hydrolysis of the input materials in order to break down insoluble organic polymers such as carbohydrates

and make them available for other bacteria. Acidogenic bacteria then convert the sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. Afterwards, acetogenic bacteria convert the fermentation products mainly into acetate. Methanogenic bacteria finally are able to convert these products to methane and carbon dioxide.

In the next sub-sections a brief description of the several consecutive biochemical steps and the different groups of specialized bacteria involved is given.

2.2.2.1 Hydrolysis

Microorganisms involved in anaerobic wastewater treatment technology cannot grab non-soluble and complex substrates that are too large to pass through cell membrane. These substrates include carbohydrates, proteins, and lipids, which are normally present in the form of suspended compounds or colloidal matter and, before transport through the cellular membrane, need to be transformed into smaller molecules. Therefore, fermentative bacteria excrete extra-cellular enzymes (hydrolases) to cleave polymers into their basic building units (monomers).

The products of the hydrolysis step are:

- Carbohydrates are converted to soluble sugars (saccharides) using cellulases;
- Proteins are degraded via (poly)peptides to amino acids using proteases and peptidases; and,
- Lipids are transformed into Long Chain Fatty Acids (LCFA) and glycerol using lipases.

In the case of complex particulate substrates, hydrolysis can be the rate limiting step in the whole degradation process (Vavilin et al., 1996; van Lier et al., 2001).

Therefore, an efficient hydrolysis step is important to make complex substrates accessible for the anaerobic bacteria, optimizing the methanogenic potential of the wastewater to be treated. The improvement of the biodegradability of particular substrate is mainly based on a better accessibility of the substrate for enzymes. In this framework, some pre-treatment techniques may be applied to enhance this step:

- Mechanical methods (Baier and Schmidheiny, 1997);
- Ultrasonic disintegration (Tiehm et al., 1997);
- Thermal pretreatment (Haug et al., 1983);
- Enzymatic and microbial pre-treatment (Hakulinen 1988); and,
- Stimulation of anaerobic microorganisms (Gossett and Belser, 1982).

2.2.2.2 Acidogenesis/Fermentation

The first energy yielding step during anaerobic digestion is the Acidogenesis (or Fermentation). During this step the hydrolysis resulting monomers (saccharides, amino acids and glycerol) are further converted inside the bacterial cells without the presence of an external electron acceptor. Normally the bacteria responsible for the hydrolysis also ferment the resulting monomers (Schink, 1997).

In general, conversion of monomers formed during the hydrolysis step results in the formation of short chain fatty acids (especially acetate, propionate, and butyrate), carbon dioxide, hydrogen, and other organic products, such as lactate and alcohols (Harper & Pohland, 1986). The several hydrolysis products are further converted:

- Soluble sugars are mainly converted to acetate and hydrogen, although it has also been observed the formation of propionate, butyrate, lactate, and ethanol (Batstone, 1999);
- The amino acids fermentation can proceed via two dominant degradation pathways: Stickland oxidation-reduction paired fermentation (require one

amino acid to act as an electron donor (oxidation) and the other to act as an electron acceptor (reduction)), or uncoupled degradation with an external electron acceptor (Batstone, 1999). The main products from amino acids degradation depend upon which of the pathways is followed. These products include, generally, short-chain and branched-chain organic acids, ammonia, carbon dioxide, and small amounts of hydrogen and sulphur-containing compounds (Ramsay and Pullammanappallil, 2001);

- Glycerol is mainly fermented to acetate, lactate and 1,3-propanediol (Biebl et al., 1999) in a biological conversion mediated by members of the *Clostridia* and *Enterobacteriaceae* groups; and,
- LCFA degradation requires an external electron acceptor for oxidation, and for that reason is covered in the acetogenesis section.

In general, the bacteria involved in this step have a short doubling time and therefore acidogenesis is not regarded as a limiting step in the process of anaerobic digestion (Gujer & Zehnder, 1983; Mosey, 1983). Organic products, including volatile fatty acids (VFA), formed during the acidogenesis are further converted by acetogens into acetate and H_2/CO_2, both of which are finally converted to methane by methanogenic archaea. However, some fermentation products, especially acetate, hydrogen, carbon dioxide and other C1 compounds, can be directly converted by methanogenic bacteria into methane and carbon dioxide.

2.2.2.3 Syntrophic Acetogenesis

Fermentation products (short chain fatty acids and alcohols) and LCFA (resulting from lipid hydrolysis) can be oxidized to acetate by the so-called obligate hydrogen producing acetogens (OHPA) or secondary-fermenting (*syntrophic*) bacteria.

Fatty acids oxidation is coupled to the reduction of hydrogen ions or bicarbonate, functioning as external electron acceptors, to hydrogen and formate, respectively. Although hydrogen appears to be an ideal electron carrier between bacteria of different metabolic types, due to its small size and easy diffusivity, formate could also act in a similar manner.

Under standard conditions, the fatty acids oxidation reactions are thermodynamically unfavourable (Table 2.1) and the complete conversion of the substrates only proceeds when hydrogen pressure in the medium is kept low. This is achieved by syntrophic association with hydrogen-utilizing bacteria, acting as hydrogen scavengers which maintain a low hydrogen partial pressure to allow for sufficient free energy (Schink, 2002). In methanogenic bioreactors, hydrogenotrophic archaea are normally responsible for the hydrogen depletion (Boone et al., 1989).

Degradation of fatty acids to acetate and hydrogen or, in the case of propionate, to acetate, hydrogen, and carbon dioxide is more endergonic under standard conditions than the ethanol oxidation (Table 2.1). Therefore, for fatty acid degradation, the hydrogen partial pressure has to be decreased to substantially lower values (<10 Pa) than with ethanol (<100 Pa) (Schink, 1997).

All organic fatty acids and ethanol are degraded by OPHA. Different pathways for the conversion of C^{4+} fatty acids, propionate, and ethanol are well reported. For full information about biochemical and microbiological aspects involved in these conversions the readers are encouraged to consult Batstone (1999), Dolfing (1988), Schink (1997), and Sousa (2007).

Table 2.1 – Gibbs free energy changes under standard conditions (adapted from Schink, 1997).

Reaction	$\Delta G^{0'}$ (kJ·mol^{-1})
Fatty acids	
$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$	+94.9
$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+76.0
$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$	+48.3
Primary alcohols	
$CH_3\ CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.6
Hydrogen-consuming reactions	
$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	-94.9
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-131.0
Syntrophic oxidation reactions	
$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36.0
$4CH_3CH_2COO^- + 4H^+ + 2H_2O \rightarrow 7CH_4 + 5CO_2$	-62.2
$2CH_3CH_2CH_2COO^- + 2H^+ + 2H_2O \rightarrow 5CH_4 + 3CO_2$	-88.5
$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COO^- + 2H^+ + CH_4 + 3CO_2$	-56.0

2.2.2.4 Methanogenesis

Methanogenesis is the final step of anaerobic degradation of organic matter. It consists in the production of biogas rich in methane. This highly specialized process is carried out by methanogenic archaea, which metabolize the end products of the previous reactions (mainly hydrogen, carbon dioxide, formate, methanol, methylamines and acetate) to form methane. In anaerobic bioreactors, this process occurs through two pathways:

- Acetate dissimilation (acetoclastic methanogenesis); and,
- Carbon dioxide reduction (hydrogenotrophic methanogenesis).

Comparing the free energies of hydrolysis of ATP (-31.8 $kJ \cdot mol^{-1}$) with those of methane formation from H_2/CO_2 and acetate (Table 2.1) shows that methanogens subsist with very low amounts of energy (Jones et al., 1987). Nevertheless, hydrogenotrophic archaea have an essential role during syntrophic acetogenesis as, maintaining hydrogen concentration at low levels, they keep the environmental redox potential at low levels.

Several authors have reported methanogenesis as being the rate-limiting conversion in the whole anaerobic digestion process in bioreactors (Fang et al., 1995; Hutnan et al., 1999; Huang et al., 2003).

Acetoclastic Methanogenesis

Acetate cleavage leads to the formation of methyl and carboxyl equivalents, which are further reduced to CH_4 and oxidized to CO_2, respectively (Jones et al., 1987). These reactions start with the activation of acetate to acetyl-CoA. The C-C bond is then cleaved by the carbon monoxide dehydrogenase system, producing HS-CoA, an enzyme-bound CO, and a methyl group. The methyl group is transferred to HS-CoM via H_4MPT. Oxidation of the enzyme-bound CO to H_2 and CO_2 provides electrons for the reduction of CH_3-S-CoM to CH_4 (Whitman et al., 1999).

Only a limited number of acetoclastic archaea has been isolated, all belonging to *Methanosaeta* (3 species, homotrophic) and *Methanosarcina* (7 species, some can also utilize H_2 and CO_2, methylated amines and methanol) genera (Elberson & Sowers, 1997; Kendall & Boone, 2004; Ma et al., 2006). Using samples from a variety of bioreactors, Zheng & Raskin (2000) demonstrated that *Methanosaeta spp.* are the dominant acetoclastic methanogens at low acetate concentrations. In the same study, *Methanosarcina spp.* outcompeted *Methanosaeta spp.* when acetate concentrations were high. These findings are in accordance with the kinetic growth parameters for these two groups, as *Methanosaeta spp.* have higher

affinities for acetate but lower growth rates than *Methanosarcina spp.* (Jetten et al., 1992).

Acetoclastic methanogens are responsible for about 70% of the total methane production in anaerobic bioreactors (Jetten et al., 1992) highlighting their importance for the efficient energetic valorisation of wastewaters.

Hydrogenotrophic Methanogenesis

The reduction of CO_2 to methane involves several steps in which the electrons source may be either H_2, via hydrogenase, or formate, via formate dehydrogenase. Initially, CO_2 is activated to form formyl-methanofuran. Next, the formyl group is transferred to H_4MPT, where it is reduced to the methylene and methyl levels. Last, the methyl group is transferred to coenzyme M and reduced to methane by the methylreductase system (Whitman et al., 1999).

Hydrogenotrophic methanogens are distributed within 5 different orders in the archaea kingdom including a large number of species (Hedderich & Whitman, 2005). In a recent survey, *Methanobacterium spp.* were identified as the most frequent hydrogenotrophs present in 44 different anaerobic digesters (Leclerc et al., 2004).

Another potential sink for hydrogen/formate is the group of homoacetogenic bacteria. Under standard conditions, methanogenic hydrogen oxidation yields more energy than homoacetogenic hydrogen oxidation (Table 2.1), therefore it is assumed that homoacetogens have little chance to outcompete successfully against methanogens for hydrogen at limiting concentrations (Schink, 1997). However, the function of homoacetogens in the complex electron flow scheme illustrated in Figure 2.1 remains unclear. Temperature is a parameter which improves the ability of homoacetogens to compete successfully against methanogens for hydrogen. At

temperatures lower than 20°C, homoacetogens appear to take over significant parts of hydrogen oxidation (Conrad and Wetter, 1990; Conrad et al., 1989). The opposite situation emerges at high-temperature habitats. Under these conditions, acetoclastic methanogenesis becomes less significant, homoacetogenesis can operate in the opposite direction, and the electron flow goes from acetate through the hydrogen toward methane (Schink 1997) (Figure 2.1).

2.2.3 The Boost of Anaerobic Wastewater Treatment Technology with Granulation

During initial experiments with an anaerobic filter, Dr. Gatze Lettinga observed that, in addition to attached biomass, a large proportion of the sludge present aggregated to form granules within the interstitial voids of the support media. This finding, in addition to the observation of a completely granular sludge in a full-scale *clarigester* in South Africa, lead Dr. Lettinga to the conclusion that inert support material for biomass attachment was not essential for retention of high levels of active sludge in the reactor (Lettinga, 2001).

Therefore, *anaerobic granules are particulate biofilms, formed spontaneously by self-immobilization of anaerobic bacteria in the absence of a support material* (Lettinga, 1995). Hence, each granule is a functional unit comprising of all the different microorganisms necessary for methanogenic degradation of organic matter (Sekiguchi et al., 1998).

The recognition of the sludge granulation concept was a significant mark in anaerobic wastewater treatment and has greatly enhanced both the efficiency and applicability of this technology. Consequently, the development of high rate reactors based in anaerobic granular sludge represented the breakthrough of anaerobic processes as a feasible and economically competitive technology for wastewater treatment. Once, the uncoupling of solids and hydraulic retention time

allowed the definitive advance for their use in a variety of industrial wastewaters (Lettinga, 1995).

The granulation phenomenon offers a vast number of advantages (McHugh et al., 2003):

- More efficient microbial proliferation;
- Access to resources and niches that cannot be utilised by isolated cells;
- Internal physicochemical gradients within the aggregates;
- Collective defence against antagonists that eliminate isolated cells;
- Optimisation of population survival by differentiation into distinct cell types;
- Continuous operation of reactors beyond normal washout rates;
- Generation of a reactor effluent with low suspended solids;
- Manipulation of biomass in a single phase; and,
- Manipulation of growth rates independent of the dilution rate.

The metabolic efficiency of cooperating communities such as anaerobic consortia depends on the efficiency of metabolite transfer between the partners involved. The flux of hydrogen between the hydrogen-forming fermenter and the hydrogen-consuming methanogen is inversely proportional to the distance between the two (Schink and Thauer, 1988). Optimal metabolite transfer can be achieved best with the two partners in close contact, *i.e.*, directly attached to each other, forming an aggregate or floc. This constitutes one more advantage of granular over suspended sludge.

However, the long start-up period required for the development of anaerobic granules limited the application of this technology. To overcome this disadvantage and apply strategies for rapid start-ups, the mechanisms for anaerobic granulation should be better understood (Liu et al. 2003). Over the last three decades,

numerous works, theories, and models have been developed to explain and/or stimulate the anaerobic granulation process (Table 2.2).

Table 2.2– Models and theories for granule formation.

Driving Force	Model/Theory	Reference
Physico-chemical	Inert nuclei	Lettinga et al. 1980
	Selection pressure	Hulshoff Pol et al. 1983
	Growth of colonised suspended solids	Pereboom, 1994
Structural/Ecological	Syntrophic microcolony	Hirsh, 1984
	Bridging of microflocs by *Methanotrix*	Dubourguier et al. 1987
	Capetown hypothesis	Sam-Soon et al. 1987
	Spaghetti	Wiegant, 1987
	Multi-layered	MacLeod, 1990
		Guiot et al., 1992
		Ahn, 2000
	Bundles of *Methanotrix enclosed by ECP*	Morgan et al. 1991
	ECP bonding	Chen and Lun, 1993
	Granulation with defined species	Wu et al. 1996
	Synthetic an natural polymer-bonding	Kalogo et al. 2001
Thermodynamic	Surface tension	Thaveesri et al., 1995
	Four-step	Schmidt and Ahring, 1996
	Crystallized nuclei formation	Zhu et al. 1997
	Proton translocation-dehydration	Tay et al., 2000
Other	Cellular automaton	Wimpenny and Colasanti, 1997
	Cell to cell communication	Davies et al., 1998
	Cluster	Gonzalez-Gil et al., 2001
	General four-step	Liu et al., 2003

Microbial adhesion or self-immobilization is the starting point of anaerobic granulation process (Liu et al., 2003). The physical theories explain this phenomenon only in terms of the physical conditions established in the reactor.

Liquid and gas up-flow velocities, suspended solids in the effluent or seed sludge, attrition and removal of excess sludge from the reactor, are considered as the key factors responsible for the phenomenon of granulation. Nevertheless, each physico-chemical model only accounts for one or two factors to the initial granulation process. Since these factors exert their influence under specific environmental conditions and in specific steps during the whole granulation process, the physico-chemical models just supply some simplified and not unified description of anaerobic granulation.

According to ecological/structural models, the characteristics of certain microorganisms involved in the degradation process are the key factor for granulation. The observation of granular characteristics, namely granule structure and correspondent microbiology, coupled to the conditions prevailing in the reactor (hydrodynamics, substrate and intermediates concentration profiles along the reactor) are the fundamentals of these theories. So far, any individual structural model cannot explain a spontaneous and sudden washout of the established granular sludge bed as a result of a change in wastewater composition, which is a common problem found in high-rate systems operation. It is a reasonable assumption that there should be a substrate composition-associated factor that highly contributes to the formation of anaerobic granules, but is not yet included in the present structural models (Liu et al., 2003).

Some authors have analysed the granulation mechanism in terms of the energy involved in the adhesion itself, due to the physico–chemical interactions between cells walls or between these and alien surfaces. Aspects like hydrophobicity and electrophoretic mobility are objectively taken into account.

Although many mechanisms and models for anaerobic granulation are currently available in the literature, none of them could provide a complete description for anaerobic granulation process. Most theories on granulation confirm that the acetotrophic methanogen *Methanosaeta* plays a key role in granulation. Some

believe that *Methanosarcina* clumps enhance granule formation. The only theory that states that other organisms cause granulation is the Cape Town hypothesis, which is based on the excessive extracellular polymers (ECP) production of *Methanobacterium strain AZ* under conditions of high H_2-partial pressures, unlimited ammonium and cysteine limitation. Nevertheless, there is still no consensus about the determining mechanism triggering granulation (Hulshoff Pol et al., 2004).

2.2.4 The Snags: Toxics and/or Inhibitors

Inhibition indicates a negative effect that a test substance causes on the activity of a microbial population. A damage on a particular bacteria function can be considered as inhibition, and, an adverse effect on bacterial metabolisms as a whole, *i.e.* in all parts of the cell functioning is described as toxicity (Rozzi and Remigi, 2004).

Two distinct patterns of toxicity are mostly reported:

- Inhibition increases as the dose of the test substance increases, but the relative activity remains constant at given times throughout the test period. This denotes a situation where the biomass does not recover from the toxic effect, *i.e.* no acclimation occurs; and,
- Temporary inhibition occurs in early stages of the test (termed, lag-phase), later on, the biomass adapts to the toxic compound by recovering its activity.

The majority of published protocols for the development of granules suggest that toxic compounds or inhibitors have a negative effect on granule development and reactors performance (Lettinga et al. 1984; Wu et al. 1987). However, with an increasing number of substrates found to be biodegradable in the absence of molecular oxygen the list of compounds not accessible by anaerobic degradation is

shrinking (Schink, 2002). A brief summary of the various compounds that have been reported to cause problems or exert toxic effects during anaerobic wastewater treatment are listed in Table 2.3.

Table 2.3 – Wastewater constituents associated with granular sludge toxicity/instability (adapted from McHugh et al., 2003).

Category	Compound and comments	Reference
Inorganic S	Sulphide (pH dependent), Sulphite	O'Flaherty et al., 1998
Aromatic	Hydrocarbons, Phenols, Chlorinated phenols, TCP Chlorobenzenes, Nitro-aromatic compounds	Fang and Chan, 1997; Christensen et al., 2004; Karim and Gupta, 2006
Aliphatic	Chlorinated aliphatic hydrocarbons (e.g. Chloroform)	Police et al., 2001; Yu and Smith, 2000
Phytochemicals	Lignin, Tannin, Wood Resin, Volatile terpenes	Sierra-Alvarez et al., 1994
Heavy Metals	Cu, Zn, Ni, Cd, Pb	Fang, 1997
Ammonia	pH dependent	Eldem et al., 2004
Salts	Na^+, K^+, Ca^{2+} (in high concentrations)	Omil et al., 1995; Feijoo et al., 1995
Organics	Formaldehyde, Azo dyes, LCFA	Gonzalez-Gil et al., 2002
Surfactants	Cationic and Anionic Detergents	Gavala and Ahring, 2002; Mensah and Forster, 2003

Acetogens and methanogens play a key role in anaerobic degradation of organic matter to methane, however, they present slow growth rates. These facts make their potential inhibition of greatest concern (Liu et al., 2003). Concomitantly, as noticeable in Figure 2.1, these bacteria are the most susceptible to inhibition. The microbial distribution of the several bacteria inside a layered anaerobic granule (see section 2.2.5) makes the aggregation a way to limit these problems. Thus, an advantage of granular biomass relative to suspended sludge is its less vulnerability

to chemical toxicity/inhibition of pollutants found in wastewater. There are several reasons for this enhanced resistance:

- A lower surface area provides better resistance against adsorbent toxins such as free LCFA (Hwu et al., 1996) and surfactants (Prats et al., 1997);
- The vulnerability of methanogens to toxins is reduced because they are mostly located in the interior of the granule, and are thus shielded by the hydrolytic/fermentative acidogens near the surface (Fang, 2000); and,
- The high intergranular carbonate and precipitated inorganic salt concentrations may allow for better buffering against pH changes during reactor overload. Methanogenic organisms (particularly *Methanosaeta*) are more susceptible to pH changes than other organisms (Boone et al., 1993).

It is also worth noting that the long biomass retention time obtained with granulation improves the tolerance of the anaerobic bacteria to the toxic compounds, allowing them to adapt to new operational conditions and possible contamination with inhibitory compounds. The high internal recirculation flow rate of high-rate reactors based in granular sludge, with emphasis to EGSB and IC reactors permit a significant dilution of the feed flow rate, and consequent decrease of possible inhibitors concentration in the influent. Since the inhibition rate of the majority of compounds increase with their concentration, the use of this kind of reactors may be essential to solve several toxicity problems.

2.2.5 Monitoring and Controlling the Process

There is a growing interest in research to develop novel tools to study, detect, and characterize microbes and their communities in industrial environments. Several analytical and investigative methods have been developed in the last years to analyse species composition, spatial structure (or architecture) and functional

properties of microbial aggregates. Simultaneously, novel methods which allow advanced mathematical modelling and numerical simulation of biological, chemical and physical processes both from reactors and aggregates became accessible.

The mutual use of several different techniques allows obtain great knowledge about the process itself and the populations and dynamics involved. Such techniques include (McHugh et al., 2003):

- Traditional enumeration methods (Kepner and Pratt, 1994);
- Microscopic: e.g. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) (Batstone, 2004; Fang, 2000);
- Immunological (Schmidt and Ahring 1999);
- Molecular biology: e.g. Fluorescent in situ hybridisation (FISH) and Denaturant gradient gel electrophoresis (DGGE) (Sanz and Köchling, 2007; Sekiguchi et al., 1999); and,
- Bacterial activity: e.g. Specific methanogenic activity (SMA) tests (Colleran et al., 1992; Coates et al., 1996).

Through the study of anaerobic aggregates, using several of this techniques, researchers (MacLeod et al. 1990; Visser et al. 1991; Guiot et al. 1992; Lens et al. 1993; Harmsen et al. 1996) suggested a layered structure of granules, in which a central core of acetoclastic methanogens is surrounded by a layer of hydrogen-producing acetogens and hydrogen-consuming methanogens, and by an outside layer of bacteria that hydrolyse and acidify complex organic matter (Figure 2.2). Fang (2000) reported the same layered structure for granules treating carbohydrate-rich wastewater. However, granules treating protein-based wastewater lack this structure (Fang et al., 1994). According to Batstone *et al.* (2004) the granule structure is highly dependent of the substrate degradation and diffusion kinetics.

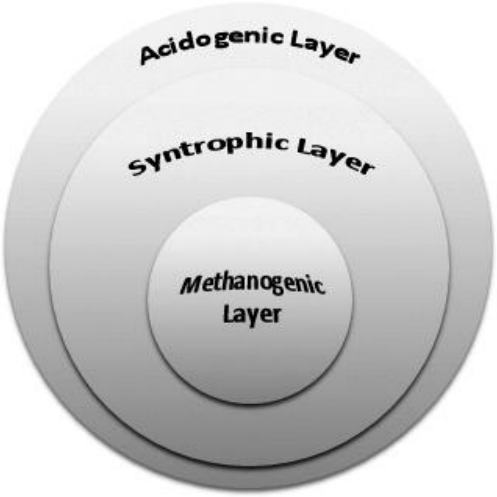

Figure 2.2 – Schematic representation of proposed layered structure of an anaerobic granule.

The novel molecular microbiology methods aim at providing insights into microscopic structures, but most of the information is of a qualitative nature. These methods have to be applied jointly with quantitative methods of modern microscopy and microsensor analysis. This will allow to directly incorporate the information gained into current design and operating guidelines and particularly to use them for model development and validation (Wilderer *et al.*, 2002).

Monitoring and process control of anaerobic wastewater treatment could be manual or completely automatic. In the past decades, a large development is going on concerning the latter approach. In this framework, both online and/or offline measurements must be available. An extensive review about instrumentation in anaerobic wastewater treatment is reported in Spanjers and van Lier (2006). An important factor for efficient operation of the anaerobic wastewater treatment technology, extensively studied in recent years, is the recognition of parameters that could be used for monitoring/control the process. It is equally feasible to obtain values of parameters measured in solid, liquid or gaseous phases. For

automatic monitoring and control, parameters in the solid phase are not often used since they usually need tedious and difficult manual operations. So far, parameters involved in reactors control had been limited to indicators of the liquid and the gaseous phases (Table 2.4). The parameters mostly used in the liquid phase are: pH, VFA, alkalinity, and COD concentrations. In the gaseous phase they are: CO_2, CH_4 and H_2 contents and gas production (van Lier et al., 2001). In this framework, quantitative image analysis techniques emerge as a promising tool to overcome these difficulties, providing quantitative parameters of the solid phase evolution.

Several works have been reported about the stability of high-rate anaerobic reactors under organic overloads or in the presence of toxic compounds. Tay and Zhang (2000a) studied the effects of stability of three high-rate anaerobic digesters (UASB, AF, AFBR) under organic and toxic shocks. It was considered that the gas phase parameters were suitable as stability indicators, although, effluent parameters, such as pH, organic concentration (COD, TOC), and VFA, should also be considered to characterize reactors status. The main control objective in anaerobic treatment plants is thus stability. However, from a control prospective, it is to be noticed that the nature of influent to be treated and the reactor configuration will largely influence the process dynamics and the achievable performances in terms of organic loading rate (Steyer et al., 2006). In Figure 2.3 is schematized the main variables used in typical control systems according to Table 2.4, where is clear that the feed flow rate (F) is the principal controlled variable.

The control systems reported in the literature can be divided in two main classes:

- Decision/Diagnostic; and,
- Control.

Table 2.4 – Synopsis of parameters used in anaerobic wastewater treatment technology monitoring and/or control and respective control systems

Reference	Reactor	Monitored	Control System	Diagnostic / Control
Garcia et al., 2007	Hybrid	[H_2], MF, IA/TA	Fuzzy Logic-based Expert System	Diagnosis / F
Pires et al., 2006	AAO	COD/N; [N-$NH4^+$]	Fuzzy Logic-based Expert System	RF, BPF
Lardon et al., 2005	CSTR	F, pH, GF, %CH_4, VFA, COD	Knowledge-based Expert System	Diagnosis
Liu et al., 2004a, 2004b	UFBR	pH, GF	Rule-based Expert System	F
Dupla et al., 2004	UFBR	F, GF, pH, %CO_2, VFA, TOC, Alk, COD	-	Diagnosis
Carrasco et al., 2002, 2004	Hybrid	GF, %CH_4, [CO], F, pH	Fuzzy Logic-based Expert System	Diagnosis
Holubar et al. 2003	CSTR	% CH_4, GF	Neural Network-based Expert System	F
Puñal et al., 2002a, 2002b	Hybrid	GF, %CH_4, [CO], F, RF, T, pH	Diagnosis Expert System	F, RF, BF / Diagnosis
Sahely and Bagley, 2001	SBR	Eff, MF; COD, Alk	Bayesian Belief Networks	Diagnosis
Puñal et al., 2001	Hybrid	F, GF, %CH_4, [CO], RF, pH, COD, Alk	Fuzzy Logic-based Expert System	Diagnosis / F
Tay and Zhang, 2000b	AFBR/AF/UASB	COD, F, Alk	Neural Fuzzy Model (Predict output variables)	VFA, TOC, MF
Huang et al., 2000	UASB	[H_2]	-	Diagnosis
Flores et al., 2000	Hybrid	T, pH, %CH_4, GF	Knowledge-based Expert System	F
Björnsson et al., 2000	CSTR	pH, Alk, VFA	-	Diagnosis
Steyer et al., 1999	AFBR	GF, Ph	-	F
Pullammanappallil et al., 1998	CSTR	%CH_4	Expert System	F
Steyer et al., 1997	AFBR	pH, T, RF, F, GF	Fuzzy Logic and Neural Networks	Diagnosis
Müller et al., 1997	AOND	GF, [H_2]	Fuzzy Inferential Control System	Diagnosis / F, BPF
Estaben et al., 1997	AFBR	GF, pH	Fuzzy Logic-based Expert System	F
Björnsson et al., 1997	CSTR	VFA, pH	-	Diagnosis
Marsilli-Libelli and Müller, 1996	AOND	GF, [H_2]	Fuzzy C-means classification	Diagnosis
Wilcox et al., 1995	AF	BA	Neural Network-based Expert System	Diagnosis
Moletta et al., 1994	AFBR	pH, GF, [H_2]	Expert System	F

Variables: %CH_4 - Methane Content in the gas phase; %CO_2 - Carbon Dioxide Content in the gas phase; [CO] - Carbon Monoxide concentration in the gas phase; [H_2] - Hydrogen Concentration in the gas phase; [N-NH_4^+] - Nitrogen Concentration in the influent; Alk – Alkalinity; BA – Bicarbonate Alkalinity; BF - Buffer Flow rate; BPF - Bypass Flow rate; COD - Chemical Oxygen Demand in the influent; COD/N – COD to N ratio in the influent; Eff - COD Removal Efficiency; F - Feed Flow rate; GF - Gas Flow rate; IA/TA - Intermediate to Total Alkalinity ratio; MF - Methane Flow rate; RF - Recycle Flow rate; T – Temperature; TOC - Total Organic Carbon; VFA - Volatile Fatty Acids; Diagnosis – applied to the detection of operational problems (Organic Overloads and/or Toxic Events).

Reactors: AAO - Integrated Anaerobic - Anoxic - Aerobic System; AF - Anaerobic Filter; AFBR - Anaerobic Fluidized Bed Reactor; AOND - Integrated Anaerobic - Aerobic - Nitrification - Denitrification System; CSTR - Continuous Stirred Tank Reactor; Hybrid - System with sludge bed at the bottom section and a filter in top (UASB + AF); SBR - Anaerobic Sequencing Batch Reactor; UASB - Upflow Anaerobic Sludge Blanket Reactor; UFBR - Up-flow Anaerobic Fixed Bed Reactor.

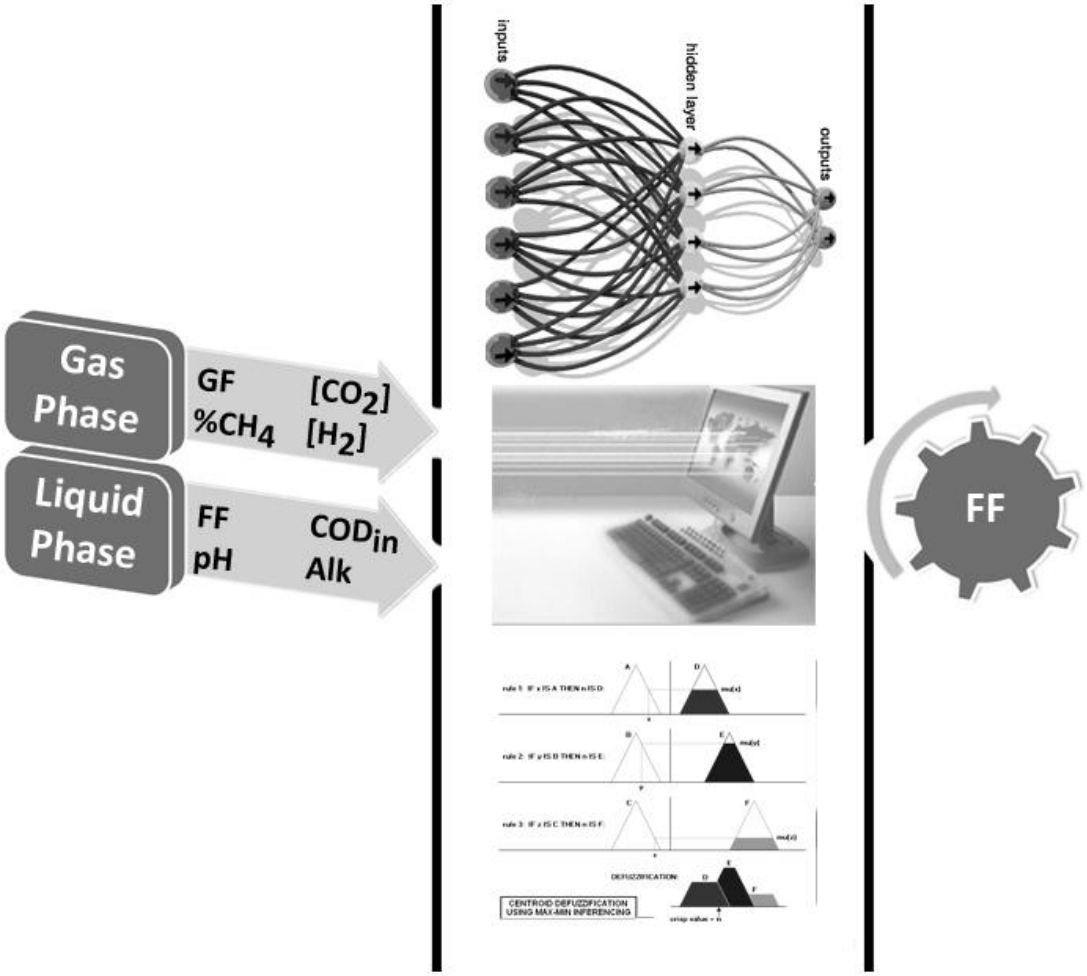

Figure 2.3 – Overall structure of a control system.

A traditional decision support system is a computer program that provides information in a given domain of application in order to support the plant manager to make effective decisions about complex and ill-structured tasks. It can be used to diagnose the state of the process as normal, overload, or toxic. The knowledge-based expert systems are more effective and apply themselves the actions needed to manage the operational problems. In this case, usually, the controlled variable is the Feed Flow rate (F) because an effective approach to dilute or concentrate the

influent, as a mean to overcome the main operational problems in wastewater treatment technology, *i.e.* overloads and/or toxics.

2.3 QUANTITATIVE IMAGE ANALYSIS

The application of image analysis procedures to complement the well established wastewater treatment operating parameters measurements is constantly growing. Both the aerobic and anaerobic processes should be of interest of this novel technique, mainly on morphological characterization of the microbial aggregates and in the evaluation of filamentous bacteria contents.

The strength of image analysis resides on the ability to remove the subjectivity of human analysis, the possibility to extract quantitative data that would be impossible or very difficult to obtain by other means, and avoid tedious and highly time-consuming tasks to human researchers (Amaral, 2003).

2.3.1 Fundamentals on Image Analysis Protocol

In this *digital era*, scarcely a week passes where we do not hear an announcement of some new technological breakthrough in the areas of digital computation and telecommunication.

This development coupled with the decrease in computers prices and increase processing and stocking capabilities, widespread these advances. Digital images are pictures that have been converted into a computer-readable binary format consisting of logical 0's and 1's. Usually, by an image we mean a still picture that does not change with time. The frequency with which information is transmitted, stored, processed, and displayed is increasing rapidly, and thus the design of

engineering methods for efficiently improve and/or employ this technology in diverse fields is of great interest.

In Figure 2.4 is schematized the main steps involved in image acquisition, processing and analysis, from the sampling until the quantitative data extraction.

The first step consists in taking a representative and homogenous sample of the object in study, through the use of a special device that would not damage the sample integrity, since microorganisms aggregates can be very unstable. A high percentage of image analysis papers describe procedures of unstained samples. However, couple image analysis with molecular techniques, such as staining or fluorescence in situ hybridization (FISH) is expanding. This technique allows a rapid quantification of several microorganisms (Hug et al., 2005).

2.3.1.1 Visualization

The first effective step in an automated image analysis protocol refers to the visualization of objects. Regarding this topic two aspects should be discussed, the type of microscope (optical or electron), and the device for image capture/treatment (off-line, on-line or in-situ).

Since the most techniques use grey images, and look just to the border-limits of the objects in study, optical microscopes are the most used to visualize samples. Standard bright-field microscope images of single or multiple cells typically contain bright objects against a darker background, making them appropriate for the most applications of quantitative image analysis. Phase-contrast lenses create a halo surrounding the objects which can give some troubles for the subsequent processing steps (Pons and Vivier, 1999). However, they are suitable for filamentous microorganisms measurements.

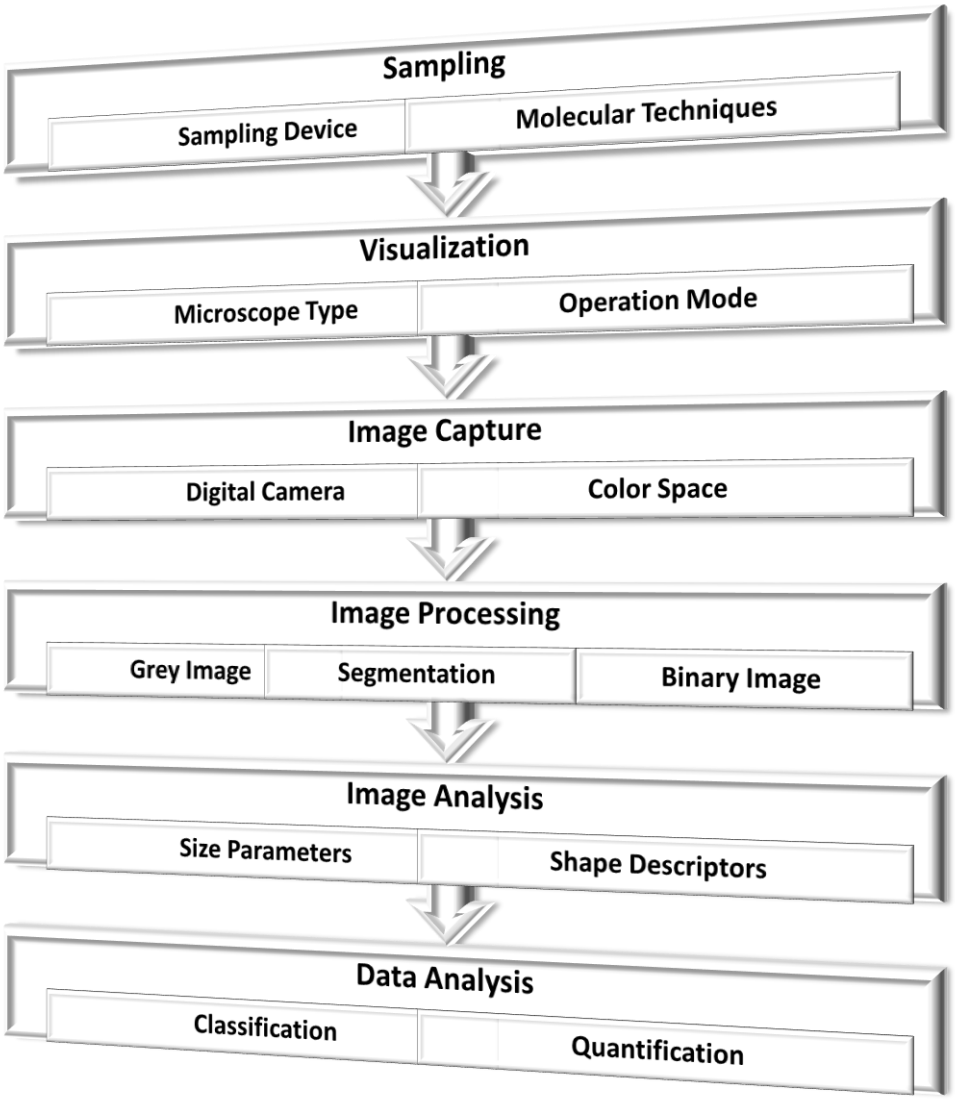

Figure 2.4 – The main steps in quantitative image analysis of microorganisms.

The use of image analysis combined with epifluorescence techniques is usually followed using confocal laser scanning microscopy (CLSM) (Kuehn et al., 1998; Schmid et al., 2003). It is particularly adapted to the study of 3D structures such as aggregates of cells (Lawrence et al., 1998).

According to Lopez et al. (2005) selection of the most appropriate technique depends on type of floc being investigated. For flocs with high cell density, the use of two proton excitation laser scanning microscopy (TPE-LSM) is preferred, since it provides a clear image of the internal structure of the aggregate. Epifluorescence microscopy did not allow to reliably quantification of red stained cells in dense aggregates. CLSM did not adequately image the internal filamentous structure and the location of stained cells within dense flocs. However, for typical activated sludge flocs epifluorescence and CLSM proved to be adequate.

Most of the actual routine image analysis applications on microorganisms are run off-line and are operator-assisted for manual sampling in the bioreactor, slide preparation (including staining) and image capture (Pons and Vivier, 1999). However, some automatic on-line systems have been reported (Govoreanu et al., 2002; Jenné et al., 2002). These systems involve circulation loops to bring the sample to the microscope. The depth-of-focus is limited and the cells should be maintained close to the focus plane by forcing them through a capillary or a flow cell (Maruhashi et al., 1994; Yu et al., 2005). Galindo et al. (2005) reported an image analysis technique for the in-situ characterization of dispersions occurring in bioreactors. However, this technique is very difficult to implement in a wide range because of equipment costs and quality of images.

2.3.1.2 Image Capture

With the amazing development of digital cameras in the last years, this step in image analysis is easily applicable. In this stage the image is captured by a digital camera and recorded in a sensor. The most used are CCD (Charge Coupled Device), and CMOS (Complementary Metal Oxide Silicon) sensors. Nonetheless, currently CCDs dominate much of the camera market because they are lightweight and cheaper (Nixon and Aguado, 2002).

The intensity of the transmitted or emitted light by the surface element corresponding to the pixel on the initial analog image is nowadays transformed into a number coded on 8 bits for monochrome images, the most widely used, corresponding to the 256 grey or brightness levels that goes from black (0) to white (255). In fluorescence images, the colour is an important factor, and the images are usually recorded in 3x8 bits. The most used colour space used is the RGB (red, green and blue channels), followed by the CMYK (cyan, magenta, yellow and black channels) and HSI (hue, saturation and intensity). Then, the image must be stored. At the moment the resolution can goes up to 12^6 pixels, however, smaller images (512x512) are usually sufficient. The uncompressed file format TIFF (tagged image file Format) is normally preferred over other compressed file format, such as JPEG (joint photographers expert group format), because of the higher image quality.

2.3.1.3 Image Processing

Subsequently to acquisition, images are then processed in order to obtain a final image, grey scaled or binary, containing information required for a given application. The first step in any method should focus on the determination and removal of the background or background light differences (Amaral, 2003).

Numerous programmes for the automatic processing of images became available in the last years. Amaral (2003) presented three specialized programmes for filaments, flocs and granules isolation and quantification. The main stages of these programmes are:

- User definitions: operator is invited to define a series of processing parameters, such as, segmentation threshold or pixel to milimeter calibration;
- Pre-treatment: resides in the grey image improvement through background correction and enhance contrast between objects and background;

- Segmentation: this step consists in transform the grey scale image into a binary image, with pure white objects (1's) and pure black background (0's);
- Debris deletion: erosion and reconstruction are used to identify and fulfil gaps inside objects; also, smaller objects and the ones cut-off by the boundaries are removed;
- Labelling: each pixel belonging to a specific aggregate is given the same discrete number differing only from aggregate to aggregate.

In the special case of filaments programme an additional step is necessary for the segmentation and elimination of aggregates. The reduced radius of gyration (Rg) is a global descriptor useful to discriminate large globular features (debris or pellets) from filamentous objects (Rg = 0.7 for a disc) (Pons and Vivier, 1999). Contreras et al. (2004) studied the competition between filamentous and non-filamentous bacteria by image analysis and concluded that objects with Roundness (Ro) < 0.38 or Rg > 1.06 should be considered filamentous microorganisms, although Rg was more accurate than Ro.

Other procedures may be found in papers reporting activated sludge characterization (Jenné et al., 2006) and semi-automatic identification of protozoa and metazoa (Amaral, 2003; Perez et al., 2006). Liu et al. (2001) developed a programme for analysis of bacteria morphotypes in microbial communities, and Daims et al. (2006) developed a novel computer programme that integrates digital image analysis and 3-D visualisation functions from epifluorescence microscopes and CLSM.

2.3.1.4 Image Analysis

In this step, normally, the final segmented binary image is used to measure several morphological parameters of the detected objects. Nevertheless, the type of

measurements determined during image analysis depends on the type of final image (binary, labelled or greyscale) and on the required data for each specific application.

The measurements obtained with this protocol can be divided in size parameters and shape descriptors. The first ones are obtained by automatically count the pixels belonging to each object, and express its size. Inside this group are the number, area, equivalent diameter, and length. Relatively to shape descriptors, it refers to morphological parameters that describe the form of the object, its circularity, elongation, etc. The shape factor, roundness, compactness, and eccentricity are between the most used, along with several others (Amaral, 2003; Pons and Vivier, 1999).

2.3.1.5 Data Analysis

This step refers to the direct application of the morphological parameters obtained with image analysis in a wide range of applications. It can be used, either for classification of the operation state of wastewater treatment plants or identification of microorganism. Also can be used to predict filamentous bulking (Bannada et al., 2005) and quantify morphological changes occurring in biomass during special events in anaerobic reactors (Araya-Kroff et al., 2004). In the next section a brief overview of image analysis applications is presented.

2.3.2 Fields of application

One advantageous aspect of image processing that makes it such an interesting topic of study is the amazing diversity of applications that use it. Virtually every branch of science has subdisciplines that use recording devices or sensors to collect image data from the universe around us (Bovik, 2000) (Figure 2.5). Afterwards,

several softwares, such as ImageJ and DAIME (Liwarska-Bizukojc, 2005), process the images to obtain the relevant data.

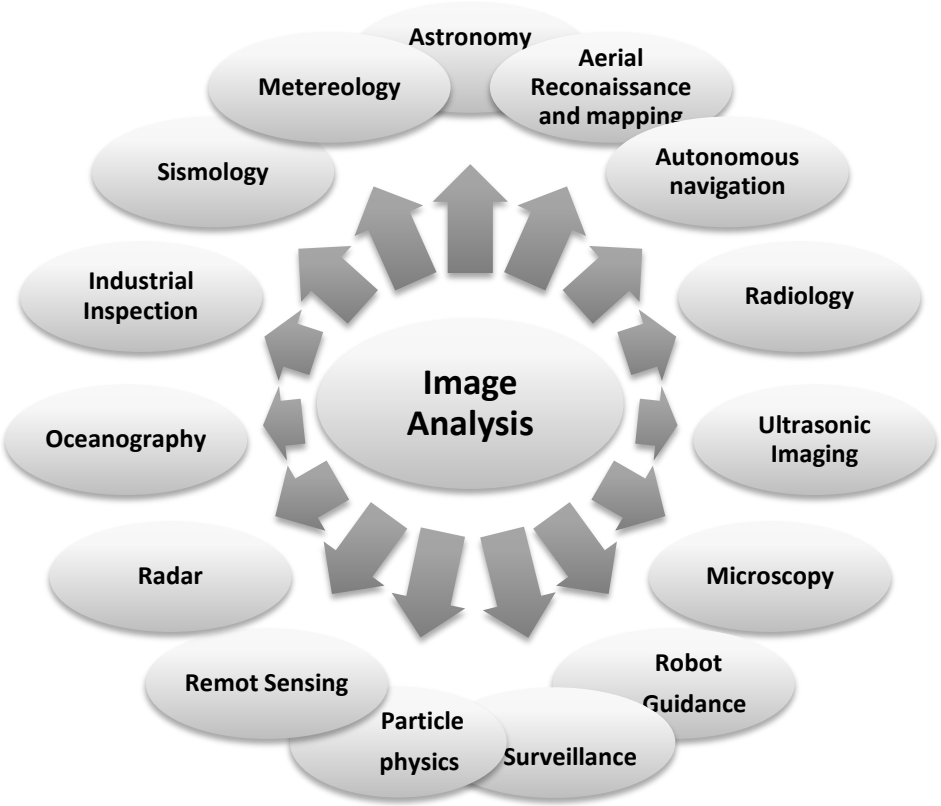

Figure 2.5 – Outline of image processing applications.

The main fields of image analysis application go from radiology, nuclear medicine and medical imaging to engineering, electrical and electronic fields. Geochemistry and geophysics, remote sensing and cell biology are other examples of areas with significant applications of quantitative image analysis techniques. Besides, biology and biotechnology, is a major field of application, such as in the study of algal cells (Martin and Fallowfield, 1989), muscle fibers (Maltin et al., 1989), plant-cells embryo (Pons and Vivier, 1998), electrophoretograms images (Horgan et al., 1992), and DNA sequencing by gel autoradiograph images.

The automated analysis of biomass now covers the whole spectrum of microorganisms: bacteria, yeast, fungi, plant and mammalian (human and animal) cells, protozoa, as well as sub-parts of cells such as RNA fragments. It has now gone beyond the stage of pure technical and mathematical development and can be used in bioresearch and in production and product-quality control.

2.3.2.1 Aerobic Wastewater Treatment Processes

Since in 1978, Mesut Segzin found that there is a well defined relationship between sludge volume index (SVI) and filament quantity (Sezgin et al., 1978), the door was opened for quantitative image analysis be applied in aerobic wastewater treatment processes monitoring and control. Since that time measurements of filaments and flocs characteristics have been made to correlate with biomass properties, with emphasis to the settleability (Sezgin et al., 1982; Andreadakis, 1993).

In Table 2.5 are summarised some key reports about applications of quantitative image analysis and respective morphological parameters used in activated sludge processes.

A succinct analysis of Table 2.5 indicates that the SVI parameter as an indicator of biomass settleability and filamentous bulking is highly correlated with the filaments contents although no general equation was yet described. Therefore, quantitative image analysis can be a powerful tool to detect this current problem in activated sludge wastewater treatment plants.

Table 2.5 – Synopsis of quantitative image analysis applications in activated sludge monitoring

References	Plant	Morphological Parameters [(1)]	Correlations / Observations [(2)]
Pandolfi et al., 2007	SBR	Number of Blue pixels	PHB storage in filaments
Smets et al., 2006	Lab-scale	AR, D_{eq}, FD, Ff, Rg, Ro, TL	Best combination for modelling the SVI: TL + FD + (Ro, Rg or AR)
Liao et al., 2006	SBR	Flocs Size	↑SRT→↑Flocs Size; ↑SVI → ↓Flocs size
Jenné et al., 2006	Lab-scale	AR, D_{eq}, FD, Ff, Rg, Ro, TL	↑SVI → ↑FD and ↑TL (PCA*)
Alinsafi et al., 2006	Lab-scale	D_{eq}, DF, Lfi, fNb	Effects of dyes: ↓(D_{eq}, Lfi, fNb)
Amaral and Ferreira, 2005	Full-scale	Conv, Ecc, Sol, TA, TL, TL/TA	↑SVI → ↑ TL/TSS; ↑TSS → ↑TA (PLS*)
Banadda et al., 2005	Lab-scale	D_{eq}, Ff, Lfi, Rg, Ro	↑(Rg, Lfi, D_{eq}, SVI) *vs.* ↓ (Ro, Ff)
Casellas et al., 2004	SBR	A, fNb, Lfi	↑SVI → ↑Lf and ↑fNb
Contreras et al., 2004	-	Rg, Ro	Filaments: Rg > 1.06 or Ro < 0.38
Jenné et al., 2003, 2004	Lab-scale	A, Ff, fNb, L, Lfi, P, Rg, Ro	↑SVI → ↑Lfi
da Motta et al., 2003	Lab-scale	D_{eq}, FD, Lfi	↑SVI → ↑Lfi
Jenné et al., 2002	-	AR, FD, Ff, Rg, Ro	Filaments/flocs: Rg most suitable
Heine et al., 2002	Lab-scale	e_{Fil}, e_{flocs}	↑SVI→ e_{fil} ; toxic inflow: ↑e_{flocs}
Govoreanu et al., 20002	SBR	CF, EF, HCF	-
Cenens et al., 2002	-	AR, FD, Ff, Rg, Ro	Filaments/flocs: Rg most suitable
da Motta et al., 2001a	Full-scale	D_{eq}, FD, fNb, Lfi, TL/TA	↑ Lfi before ↑SVI
Grijspeerdt & Verstraete, 1997	-	AR, D_{eq}, FD, Ff, Ro	↑SVI → ↓Ff
Ganczarczyk, 1994	Full-scale	A, D_{eq}, L, P	↑v_{sed} → ↓D_{eq} and ↓L

(1) A – Aggregates area; AR – aspect ratio; CF – compactness factor; Conv – Convexity; D_{eq} – aggregates equivalent diameter; Ecc – Eccentricity; e_{Fil} – filamentous fraction; e_{flocs} – flocs fraction; EF – Elongation factor; FD – fractal dimensions; Ff – form factor; fNb – filaments number; HCF – Heywood circularity factor; L – aggregates length; Lfi – filaments length by image; P – aggregates perimeter; Rg – reduced gyration radius; Ro – roundness; Sol – Solidity; TA – total aggregates area; TL – total filaments length; TL/TA – ratio between total filaments length and total aggregates area; W – aggregates width.

(2) SVI – sludge volume index; SRT – sludge retention time; PHB – poly-β-hydroxybutyrate.

* Correlations detected by multivariate statistical analysis: PCA – principal components analysis; PLS – partial least squares regression.

Another field of application of image analysis in activated sludge processes is the identification of Protozoa and Metazoa populations, which are between the most important indicators of activated sludge state (Madoni, 1994). Coupled with multivariate statistical techniques, such as principal components analysis (Amaral et al., 1999; da Motta et al., 2001b) and discriminant analysis (Amaral et al., 2004a), morphological parameters can be used to recognized above 80% of protozoa and metazoa species. Ginoris et al. (2007a) showed that the use of discriminant analysis and neural networks was more appropriate than decision trees in identification of 22 classes. It is important to enhance that all this procedure takes about 2 or 3 hours (Ginoris et al., 2007b).

Image analysis techniques have also been applied to membrane reactors, mainly to study the structure of biofilms and correlation with membrane permeability (Meng et al., 2006; Yun et al., 2006).

2.3.2.2 Anaerobic Wastewater Treatment Processes

During the last decades, with the rising of anaerobic granular sludge in wastewater treatment processes spot, the study of structural changes in terms of size, strength, density, and settleability of anaerobic microbial aggregates is growing. Several direct and indirect methods to quantify the physical characteristics of the anaerobic granular sludge have been presented during last decades (Ahn and Speece, 2003):

- Direct granular particle *size* analysis was performed manually with a portion graticule/lattice ruler (Hulshoff Pol, 1989) or wet sieving using phosphate buffer solution or tap water (Laguna et al., 1999), and by particle size analysis using a laser (Yan and Tay, 1997);
- The *strength* of granules was measured in several studies by examining the effects of shear force, sonication, shaking of the granules, or turbidity (Teo et al., 2000; Quarmby and Forster, 1995);

- Indirect granular sludge *density* analysis was performed by the measurement of the settling velocities of a sludge sample to extrapolate the corresponding diameters (Hulshoff Pol, 1989; Grotenhuis et al., 1991) or SVI (Ahn, 2000; Cuervo-López et al., 1999; Yan and Tay, 1997);
- The *settleability* profile analysis performed using an upflow-type settling column (Ahn, 2000; Andras et al., 1989).

Morphological parameters may be helpful in the monitoring of anaerobic granular sludge stability during transient processes. The first attempts to use digital image analysis in anaerobic wastewater treatment processes were limited to size measurements and number counting (Dudley et al., 1993). Since in 1998, Jeison and Chamy (1998) presented a novel technique for measuring the size distribution of granules using a scanner to acquire digitalised images of samples embedded in gelatine, ample specialized programmes/protocols became available to measure, in a straightforward way, the physic characteristics of the biomass. In the field of wastewater treatment technology, the works reported using quantitative image analysis (Table 2.6) goes from the simple differentiation between flocs and granules (Bellouti et al., 1997), to the characterisation of hydrogen-producing granules (Mu and Yu, 2006b). The definition of special morphological indicators relating the filamentous contents and aggregates area allowed an early detection of washout events (Amaral et al., 2004b) and the biomass aggregation time (Araya-Kroff et al., 2004). Relationships between morphological parameters and specific methanogenic activity were also reported (Alves et al., 2000; Abreu et al., 2007).

Another techniques, coupled with special microscopes and molecular techniques had been achieved. Howgraves-Graham and Wallis (1993) used TEM to study the inner structure of aggregates. And, based on the auto-fluorescence of methanogenic bacteria other approaches can be considered (Ahn et al., 2000).

Table 2.6 – Synopsis of quantitative image analysis applications in anaerobic wastewater treatment processes.

References	Reactor	Role of Image analysis	Morphological Parameters [(1)]	Correlations / Observations
Abreu et al., 2007	EGSB	Structural changes during SAA recovery	%Area, LfA, TL/VSS, VSS/TA	Onset biogas production with ↑TL/VSS
Mu and Yu, 2006a	UASB	Fractal dimensions of granular sludge	FD	FD = 2.79 ± 0.03, rheological *vs.* Df characteristics
Mu and Yu, 2006b	UASB	Morphology of H_2-producing granules	%Nb, d, FD	d = 1.0 – 3.5 mm, FD = 1.78
Amaral et al., 2004b	EGSB	Oleic Acid effects on biomass morphology	%Area, %Nb, LfA	LfA as early-warning of washout
Araya-Kroff et al., 2004	EGSB	Quantify granulation process	%Area, D_{eq}, LfA, TL/VSS, VSS/TA	LfA to detect granulation time
Singh & Viraraghavan, 2003	UASB	Impact of Temperature on granulation	D	↓T → ↑d
Pereira et al., 2003	EGSB	Effects of ↑[Oleic Acid] in morphology	D_{eq}	↑ [Oleic Acid] → ↓D_{eq}
Ahn & Speece, 2003	UASB	Validate settleability protocol	%Nb	↑ vs → ↓v_{sed} and D_{eq}
Alves et al., 2000	UFBR	Filaments changes during organic shocks	fNb, TL	fNb and TL ↑ → SAA ↑ (low [substrate])
Jeison & Chamy, 1998	-	Granules size distribution	%Area	-
Singh & Viraraghavan, 1998	UASB	Biomass granulation during star-up at 20ºC	d, Nb	↓HRT → ↑d ; ↓Nb → aggregation
Bellouti et al, 1997	UFBR UASB	Flocs *vs.* Granules	FD (Box counting / Power law)	FD (Flocs) = 1.90 ± 0.02 / 1.84 ± 0.13 FD (Granules) = 1.95 ± 0.01 / 2.14 ± 0.08
Dudley et al., 1993	UASB	Characterize anaeerobic granules	Number, Size and Density	-

(1) **%Area** – Total aggregates projected area distribution by equivalent diameter ranges; **%Nb** – total number of aggregates distribution by equivalent diameter ranges; **d** – aggregates diameter, **D_{eq}** – equivalent diameter; **FD** – fractal dimension; **fNb** – Free filaments number; **LfA** – total filament length per total aggregates projected area; **Nb** – Number of aggregates; **TL** – total filaments length; **TL/VSS** – total filament length per volatile suspended solids; **VSS/TA** – volatile suspended solids per total aggregates projected area

Summarising, the quantitative image analysis is mainly used in anaerobic wastewater treatment processes as a tool to detect morphological changes in the biomass when sudden changes in reactor normal operation occur. The determinations of filaments length, and aggregates area and diameter are the main indicators used in this field of application.

2.4 MULTIVARIATE STATISTICAL ANALYSIS

The proliferation of instrumentation in environmental processes capable of rapidly producing vast amounts of data, and the widespread availability of powerful, inexpensive computers set up the conditions for the rapid progression of chemometrics methods over the last decades. The basic concept of processes monitoring is schematized in Figure 2.6. It is commonly accepted that as sooner the problem is identified, better will be the implementation/efficiency of corrective actions. Consequently, over the past years we assist to the instrumentation and automation of many processes. However, gathering large amounts of data from highly automated processes guide analysts to concomitantly assemble important and redundant or noisy information. Multivariate statistical methods are important tools that may/should be used to extract the latent information from these data.

Application of chemometric methods may be very useful for the solution of the following environmental problems (Einax et al., 1997):

- Planning and optimization of the whole environmental analytical process, starting with the planning and experimental design of environmental sampling or experiments, optimization of the analytical procedure in the laboratory, including modern chemometric methods for signal detection and treatment;

- Compression of large data sets eliminating redundancy and noise;
- Visualization of complex, often high-dimensional quantitative relationships;
- Detection and identification of emitters and dischargers (in general: origins);
- Detection and quantification of differences in loadings (*i.e.* variables weights);
- Investigation of spatial and temporal relationships between data and their changes;
- Investigation of different species in the environment;
- Investigation of interactions between pollutants and components of environmental compartments; and,
- Environmental impact assessment.

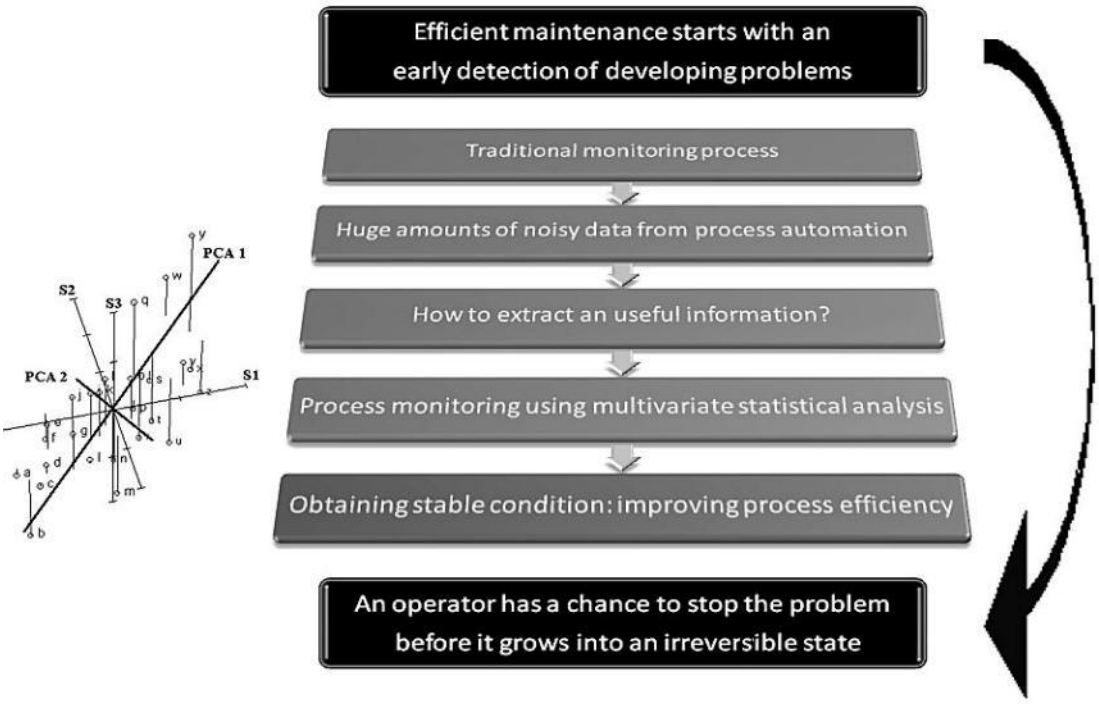

Figure 2.6 – Concept of process monitoring for efficient operation.

Furthermore, with an earlier recognition of operational problems it is possible to take corrective actions to minimize or overcome the problem (Figure 2.6).

Chemometrics methods range from simple statistics to highly sophisticated data analysis. In Table 2.7 are summarised important multivariate statistical analysis methods, as well as typical problems where they can be applied.

Table 2.7 – Key methods of multivariate statistical analysis (adapted from Einax et al., 1997).

Method	Solving the problem
Unsupervised learning methods	
Cluster analysis Display methods *Nonlinear mapping (NLM)* *Minimal spanning tree (MST)* *Principal components analysis (PCA)*	Finding structures/similarities (groups, classes) in the data
Supervised learning methods	
Multivariate analysis of variance and discriminant analysis (MVDA) k nearest neighbours (kNN) Linear learning machine (LLM) BAYES classification Soft independent modelling of class analogy (SIMCA) UNEQ classification	Quantitative demarcation of a priori classes, relationships between class properties and variables
Factorial methods	
Factor analysis (FA) Principal components analysis (PCA) Partial least squares modeling (PLS) Canonical correlation analysis	Finding factors (causal complexes)
Correlation and regression analysis With direct variables With latent variables	Quantitative description of the relationships between variables

The chemometric techniques are designed to extract useful information from very subtle differences in the data (Kramer, 1998). Typical questions when analysing a dataset, *i.e.* a matrix of *m* objects (or observations) per *n* variables:

- Are there relationships between objects?
- Are there relationships between variables?
- Are there *dependencies* of (grouped) objects?
- Are there dependencies of one or several variables on one or several others, and can they be modelled?

Unsupervised learning or unsupervised pattern recognition methods are more appropriated to answer the two first questions. These methods may also be called grouping methods or automatic classification methods because they search for classes of similar objects (e.g. cluster analysis) or classes of similar features (e.g. correlation analysis, factor analysis). Besides, the third question is adequate for the supervised learning methods, and regression methods are designed for the fourth question (Einax et al., 1997).

2.4.1 Principal Components Analysis

Principal Components Analysis (PCA) is a favourite tool of chemometricians for data compression and information extraction (Wise and Gallagher, 1996). It finds combinations of variables, or factors, which describe major trends in the data. Mathematically, PCA relies upon an eigenvector decomposition of the covariance or correlation matrix of the data variables (Jackson, 1980).

The principle of PCA is the transformation of the original features into uncorrelated new variables (principal components) by means of linear combinations. The coefficients (loadings) are chosen so that the new variables, unlike the original variables, are not correlated with each other. Creating a new set of variables in this way may seem a pointless exercise since we obtain *n* new variables in place of the *n* original ones, and hence no reduction in the amount of data. However, the principal components (PCs) are also chosen so that the first accounts for most of the

variation in the dataset, the second accounts for the next largest variation and so on. Thus, when significant correlation occurs, the number of useful PCs is much less than the number of original variables (Miller and Miller, 2000). It is often found that PC1 and PC2 account between them for the most and more important variation in the dataset. As a result the data can be represented in only two dimensions instead of the original *n*.

In the last two decades several statistical methods for process monitoring have been applied to wastewater treatment plants. The most common application of PCA is statistical process control. Rosén and Olsson (1998) show disturbance detection in wastewater treatment system using PCA and PLS. Raich and Çinar (1996) propose to utilize multivariate statistical techniques (PCA and discriminant analysis) to detect states out of control and to diagnose abnormal operation conditions and disturbances that cause poor process efficiency. Tomita et al. (2002) performed analysis of an activated sludge process using PCA. It shows a method to extract information for easy interpretation, disturbance or deviation detection of normal condition in measurements of wastewater treatment process, and, suggest the variables responsible for the deviations. Recently, this technique was successfully applied in the surface water quality monitoring of the Fuji river basin (Japan) (Shrestha and Kazama, 2007), and, the levels of polychlorinated dibenzo-p-dioxins and dibenzofurans in 31 sewage sludges in the Valencian Community (Spain) (Fuentes et al., 2007).

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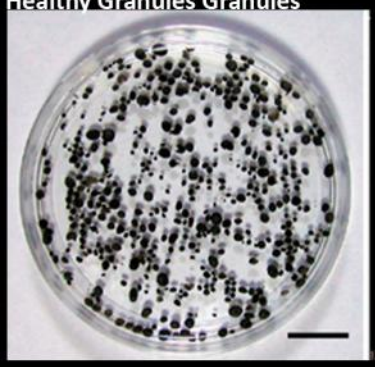
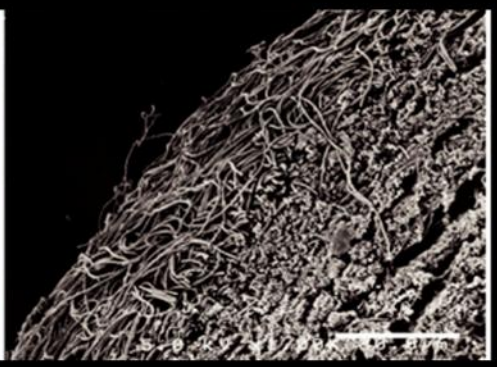
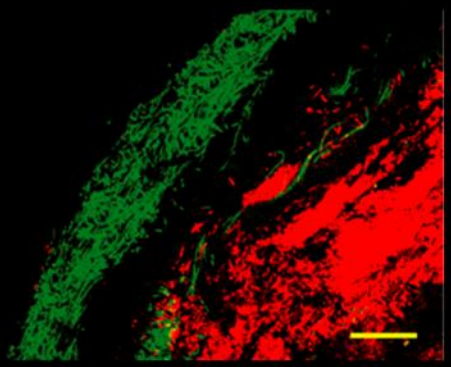
Healthy Granules Granules

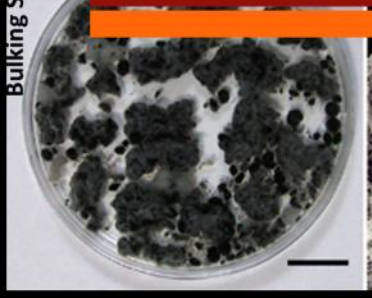


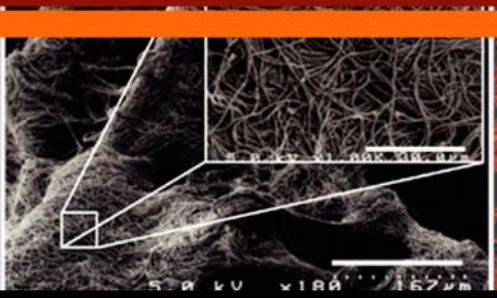


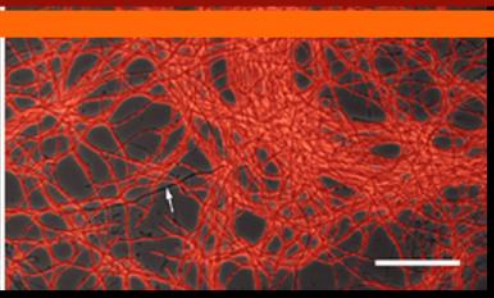


Methodology

Bulking Sludge Granules







ABSTRACT

In this section the experimental set-up, operating parameters used during routine analysis, the specific methanogenic activity methodology, image analysis technique, and principal components analysis are presented.

A lab-scale Expanded Granular Sludge Bed Reactor was used to apply transient disturbances in a typical anaerobic granular sludge process. Specific Methanogenic Activity assays were performed to verify the effects in the biomass physiology. And, an image analysis technique was used to quantify the morphological changes during those disturbances. This section presents the morphological parameters and a brief description of the programmes used for the determination of those indicators.

A multivariate statistical analysis, called Principal Components Analysis, was used to find patterns in data and look for correlation between the several parameters monitored. A brief description of this technique is given.

3.1 EXPERIMENTAL SET-UP

In the experiments presented in this thesis an Expanded Granular Sludge Bed (EGSB) reactor (Figure 3.1) was used. It consisted in a Plexiglas column with a height of 1.95 m and internal diameter of 21 mm. The working volume was 1.15 L and the up-flow velocity was 4.0 $m.h^{-1}$. Temperature was kept at 37 ± 1 ºC by means of an external jacket for water circulation.

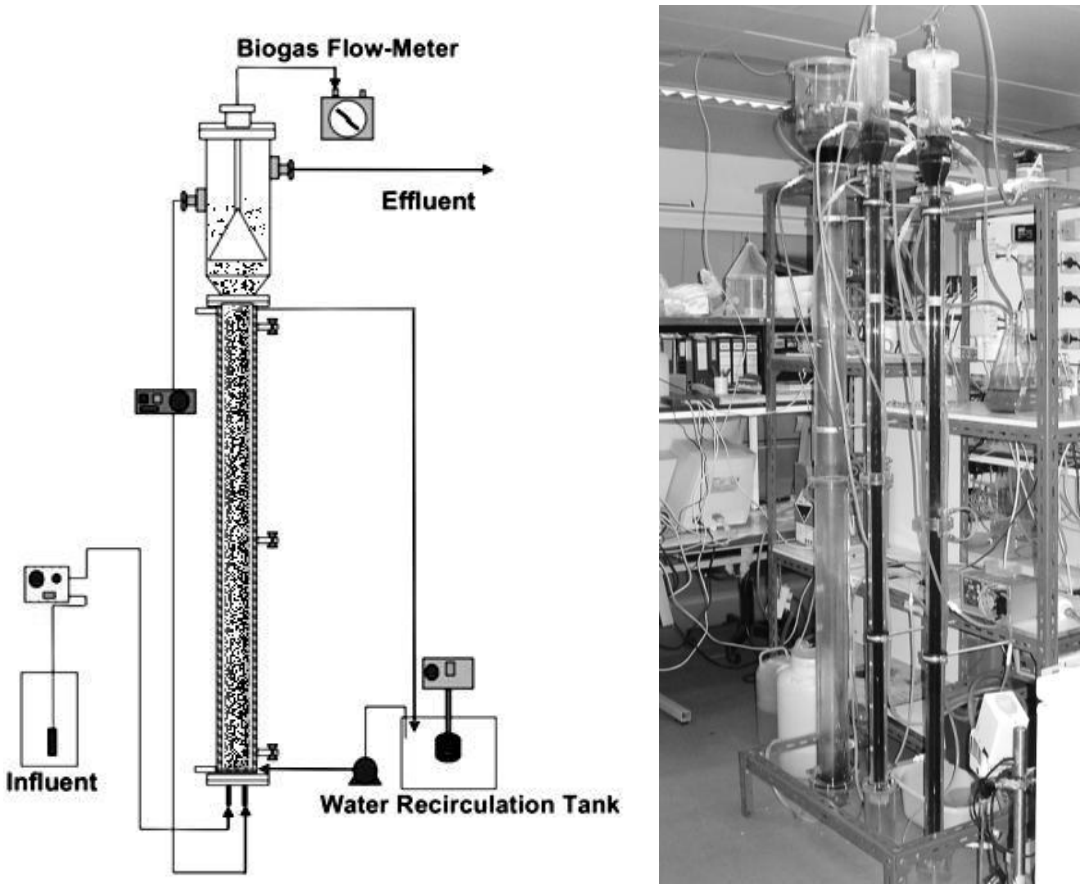


Figure 3.1 – Expanded Granular Sludge Bed Reactor.

3.2 OPERATING PARAMETERS

Several operating parameters were determined by routine analysis during EGSB reactor operation. Brief descriptions of those parameters are presented in the next paragraphs.

- The *Chemical Oxygen Demand* (COD) and *Volatile Suspended Solids* (VSS) were determined according to Standard Methods (APHA, 1989).
- *Biogas flow rate* was measured by a *Ritter Milligascounter* (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany).
- *Methane content of biogas* was determined by gas chromatography using a *Porapack Q* (100 - 180 mesh) column, with Helium as the carrier gas at 30 $mL \cdot min^{-1}$ and thermal conductivity detector. Detector, injector, and oven temperatures were 110, 110, and 35 ºC, respectively.
- *Volatile fatty acids* (VFA) and *ethanol* were determined by high performance liquid chromatography using an *HPLC* (Jasco, Japan) with a *Chrompack column* (6.5 x 30 mm^2); sulfuric acid (0.01 N) at a flow rate of 0.7 $mL \cdot min^{-1}$ was used as mobile phase. Column temperature was set at 60 ºC. Detection of VFA and ethanol was made sequentially with an UV detector at 210 nm and a RI detector, respectively.
- The *Hydraulic Retention Time* (HRT) is the average time spent by the influent liquid inside the reactor and is determined by:

  $$HRT = \frac{V}{F} \quad (3.1)$$

  Where, *V* is the reactor volume and *F* is the feed flow rate.
- The *Organic Loading Rate* (OLR) is the COD flow rate and is determined by:

  $$OLR = \frac{COD}{HRT} \quad (3.2)$$

- The *Up-flow Velocity* is the liquid velocity the inside the reactor, and is given by:

  $$v = \frac{F+RF}{S} \quad (3.3)$$

  Where, *S* is the reactor Section Area, *F* is the Feed Flow Rate, and *RF* is the Recycle Flow Rate.

3.3 BIOMASS SAMPLING

One of the most critical steps in this methodology is the sludge sampling. Effectively, it is imperative to preserve the biomass integrity and do not provoke changes in morphological characteristics of the granular sludge. A sampling device was used to take biomass from the reactor without disturbing its morphology. It was introduced at the top of the reactor and biomass was collected to the tube, along the reactor to get homogeneous and representative sample, avoiding mechanical stress. All the sludge samples were characterized by image analysis, specific methanogenic activity assays, settling velocity, and volatile suspended solids content.

3.4 SPECIFIC METHANOGENIC ACTIVITY

Specific Methanogenic Activity (SMA) assays were performed using a pressure transducer technique (Colleran *et al.*, 1992). The Specific Acetoclastic Activity (SAA) was measured in the presence of acetate (30 mM) and the Specific Hydrogenotrophic Methanogenic Activity (SHMA) was measured in the presence of H_2/CO_2 80:20 v/v, at 1 bar.

The pressure increase developed in the batch vials fed with nongaseous substrates (acetate), or pressure decrease in vials previously pressurized (1 bar) with gaseous substrates (H_2/CO_2) was monitored, using a hand-held pressure transducer capable of measuring a pressure variation of 2 bar (0 to ±202.6 kPa) over a device range of -200 to +200 mV, with a minimum detectable variation of 0.005 bar, corresponding to 0.05 mL of biogas in a 10 mL headspace.

The basal medium used in all batch experiments was made up with demineralised water, was composed of cysteine-HCl (0.5 $g \cdot L^{-1}$) and sodium bicarbonate (3 $g \cdot L^{-1}$), the pH was adjusted to 7.0–7.2 with NaOH (8 N), and was prepared under strict anaerobic conditions. No nutrients were added.

Methane content of biogas was determined by gas chromatography using a *Porapack Q* (100-180 mesh) column, with Helium as carrier gas at 30 $mL \cdot min^{-1}$ and a flame-ionization detector. Detector, injector, and oven temperatures were 110, 110, and 35 ºC, respectively. The values of methane production were corrected for the standard temperature and pressure conditions (STP).

Blank controls were used for liquid substrate (no added substrate) and for gaseous substrate (pressurized with N_2/CO_2, 80:20 v/v at 1 bar). All batch experiments were performed in triplicate.

SMA values were determined dividing the initial slope of the methane production curve by the VSS content of each vial at the end of the experiment and were expressed in $mL_{CH4(STP)} \cdot g_{VSS}^{-1} \cdot d^{-1}$. Background methane production due to the residual substrate was discounted.

3.5 IMAGE ANALYSIS TECHNIQUE

3.5.1 Dilution

Biomass samples must be diluted for image analysis using an optimized dilution factor. When the dilution is excessive the observer may unconsciously search objects over estimating them. If the dilution is insufficient, the objects will be overlaid. The optimal dilution value was determined as the lowest dilution that enabled the maximum percentage of objects to be recognized. The percentage of

recognition is the ratio between the area of objects that are completely inside the image and the total area of objects in the image including those that are at the boundaries and cannot be completely recognized. In these experiments, the optimal dilution varied between 1:5 and 1:10.

3.5.2 Image acquisition

For the acquisition of filaments and micro-aggregates (Equivalent Diameter (D_{eq}) < 0.2 mm) images, a volume of 35 µL from the diluted sample was distributed on a slide and covered with a 20x20 mm cover slip for visualization and image acquisition. This volume was exactly covered by the cover slip. Each image corresponded to a volume of 0.0445 µL. Subsequently, more than 120 images were acquired. Image acquisition was obtained by dividing the cover slip into 42 identical fields and taking a photo in each imaginary square (Figure 3.2). At least three slides were examined to minimize sampling errors.

Concerning to macro-aggregates (D_{eq} ≥ 0.2 mm) images, an arbitrary volume was transferred to a Petri dish for visualization and image acquisition. All the aggregates present in that volume were *digitalised* (Figure 3.3). A minimum of 120 images were captured. Then, the VSS content in the Petri dish was measured in order to standardize the measurements in each sample by its VSS.

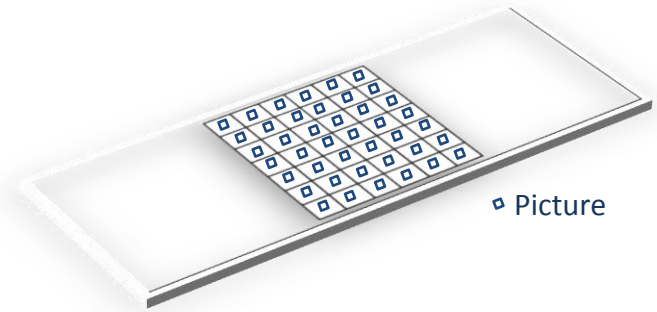


Figure 3.2 – Image acquisition methodology for filaments and micro-aggregates determination.

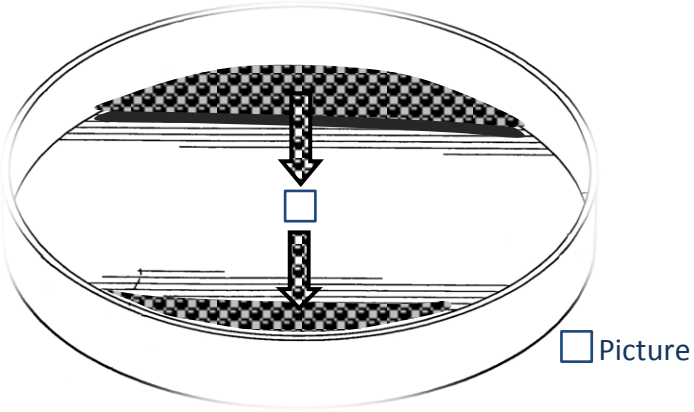


Figure 3.3 – Image acquisition methodology for macro-aggregates determination.

Images used to quantify filaments and micro-aggregates were acquired through phase contrast and bright field, respectively, on a *Nikon Diaphot 300* microscope (*Nikon Corporation, Tokyo*) with 100x magnification. Images used to quantify macro-aggregates were acquired through visualization on an *Olympus SZ 40* stereo microscope (*Olympus, Tokyo*) with 15x magnification. All the images were digitized and saved with the help of a *CCD AVC D5CE Sony* grey scale video camera (*Sony, Tokyo*) and a *DT 3155 Data Translation* frame grabber (*Data Translation, Marlboro*) with 768 x 576 pixel size in 8 bits (256 grey levels) by *Image Pro Plus* (*Media Cybernetics, Silver Spring, MD*) software package.

3.5.3 Image processing and analysis

Image processing and analysis was accomplished by means of three programmes developed in *Matlab* (*The Mathworks, Inc., Natick*), for filaments, micro, and macro-aggregates (Amaral, 2003). Brief descriptions of the programs are presented in the next subtitles.

3.5.3.1 Filaments Programme

In this programme the grey-scale image (Figure 3.4a) was first divided by the background image in order to remove background light differences. A bottom hat filter (Russ, 1995) was then applied to enhance the filaments and small aggregates that have low grey levels. The larger aggregates, which have high grey levels, were subsequently identified on the image resulting from the background elimination step by applying a 10-order closing (to enhance the aggregates), a segmentation at a fixed threshold value, a filling of the resulting binary image (to remove the inner holes in the aggregates) and a erosion-reconstruction step to eliminate the debris. Filaments and small aggregates were then isolated by segmentation at a fixed threshold and by logic subtraction of the mask binary image containing the large aggregates. Then, the small aggregates were eliminated by deleting all the objects smaller than 32 pixels (in area) or with a gyration radius below 1.2 (Pons and Vivier, 1999). The final image (Figure 3.4b) contained only filaments that were characterized in terms of their length and number. The filaments image was skeletonised and pruned (Russ, 1995).

Filaments are not only the dispersed bulk filaments, but include also those that are attached to an aggregate and still have one free extremity (protruding filaments) (Figure 3.4a).

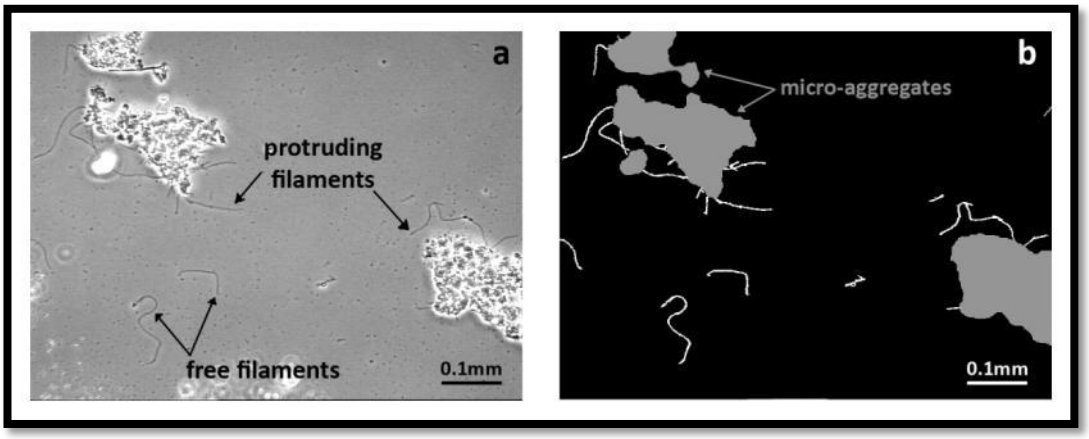


Figure 3.4 – Original (a) and binary (b) images of filaments.

3.5.3.2 Micro-aggregates Programme

In this programme the grey-scale image (Figure 3.5a) was first divided by the background image to remove background light differences. Then, a histogram equalisation was performed to enhance the micro-aggregates followed by an image smoothing Wiener filtering (Glasbey and Horgan, 1995). The image was then segmented in black (background) and white (objects) by the simultaneous use of a boundary based segmentation and a user chosen or automatically determined threshold segmentation. The objects smaller than 3x3 pixels (small debris) were then removed and small gaps (6x6 pixels or less) were filled on the remaining objects. Subsequently, in order to remove filaments, all the objects smaller than 2000 pixels in area and with a gyration radius above 1.2 (Pons and Vivier, 1999) were deleted. Finally, all the objects cut off by the image boundaries were removed (Figure 3.5b), and the morphological characterization of the micro-aggregates was performed.

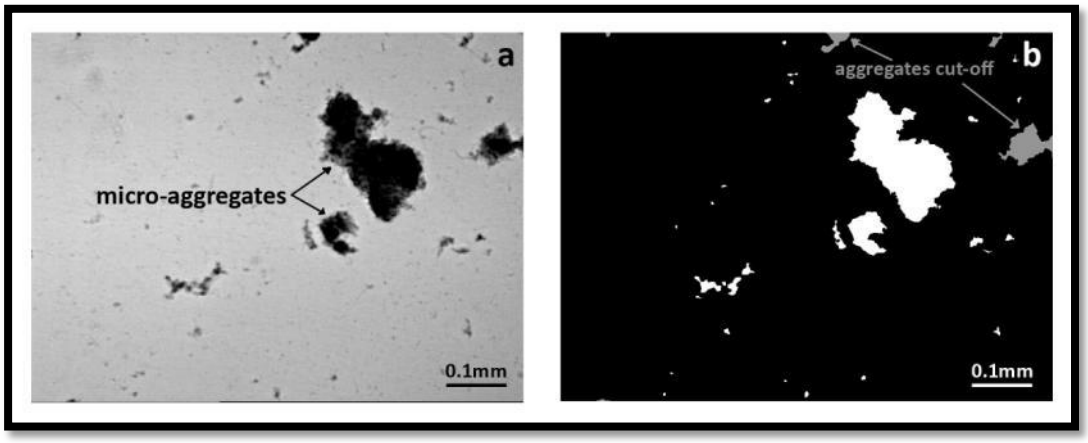


Figure 3.5 – Original (a) and binary (b) images of microflocs.

3.5.3.3 Macro-aggregates Programme

In this programme the grey-scale image (Figure 3.6a) was first divided by the background image in order to remove background light differences. Next, the image was smoothed by Wiener filtering (Glasbey and Horgan, 1995). The image was then segmented in black (background) and white (aggregates) by threshold segmentation, with a user chosen or automatically determined threshold. Subsequently, small gaps (6x6 pixels or less) were filled on the objects, and small debris (3x3 pixels or less) were removed. Finally, all the objects cut off by the image boundaries were removed, and the morphological characterization of the macro-aggregates was performed from the final binary image (Figure 3.6b).

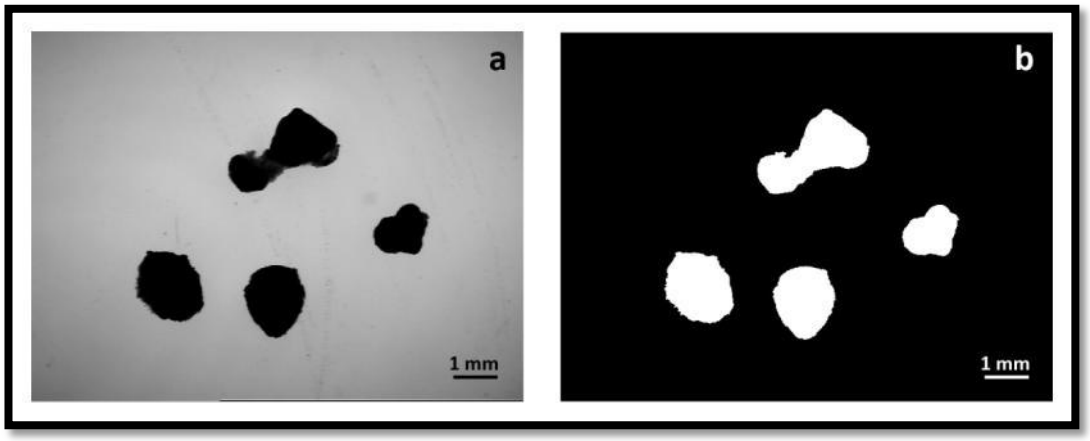


Figure 3.6 – Original (a) and binary (b) images of macroflocs.

3.5.3.4 Morphological parameters

The second part of the programmes calculates several morphological parameters that are saved in a TXT (Text File) format file. The main parameters determined are summarised in the next paragraphs.

- The *Filaments Length* (*L*) was determined by:

$$L = N \times 1.222 \times F_{cal} \quad (3.1)$$

Where, *N* is the number of pixels of the skeletonized filament and F_{cal} is the calibration factor (µm·pixel^{-1}) obtained using a micrometer. The factor 1.122 is used in order to homogenize the different angles of the filaments (Walsby and Avery, 1996).

- The *Specific Total Filament Length* (L_{spec}) was calculated as:

$$L_{spec} = \frac{\sum L}{V_{field}} \quad (3.2)$$

Where, V_{field} is the volume (in µm^3) corresponding to the field of view (*i.e.* the image).

- The *Aggregate Area* is calculated as the projected object surface and is given by the number of pixels belonging to an object converted to metric units:

$$Area = N_{obj} \times F_{cal} \quad (3.3)$$

where N_{obj} is the pixel sum of any individual object, and F_{cal} is the calibration factor (µm.pixel^{-1}) obtained using a micrometer.

- The *Equivalent Diameter* (D_{eq}) is then calculated as:

$$D_{eq} = 2 \times \sqrt{\frac{Area}{\pi}} \quad (3.4)$$

- The *Total Area* (A_T) is given by the cumulative *Area* of all aggregates including the ones cut off by the image boundaries. Then, the *Specific Area* occupied by aggregates (A_{spec}) is calculated as:

$$A_{spec} = \frac{A_T}{V_{field}} \quad (3.5)$$

- Morphological parameters representing the dynamic evolution of filaments and aggregates inside the reactor were calculated as:

$$TL/VSS = \frac{L_{spec}}{VSS} \quad (3.6)$$

$$VSS/TA = \frac{VSS}{A_{spec<0.2mm} + A_{spec\geq 0.2mm}} \quad (3.7)$$

Where, *VSS* are the Volatile Suspended Solids present in each sample, and $A_{spec<0.2mm}$ and $A_{spec\geq 0.2mm}$ are the specific aggregate area ratio for aggregates with D_{eq} < and ≥ 0.2 mm, respectively.

- A morphological parameter based on the ratio of specific total filament length to total aggregates projected area (*LfA*) is determined by:

$$LfA = \frac{L_{spec}}{A_{spec<0.2mm} + A_{spec\geq 0.2mm}} \quad (3.8)$$

- The *%Area* of each size class is given by the ratio between the sum of the *Aggregate Area* of that size class and the *Total Area*:

$$\%Area = \frac{\sum_{i=1}^{n_{class}} Ai}{TA} \quad (3.9)$$

Where, *Ai* is the *Area* of each *i* aggregate belonging to that size class.

3.6 SETTLING VELOCITY

The settling velocity (v_{sed}) was measured by depositing several biomass samples in the top of a column filled with water. Afterwards the time that each particle takes to cover the 320 mm of the column was measured using a chronometer that automatically registers that time in an Excel spreadsheet. Then the average of all individual sedimentation velocities was determined. More than 150 particles were considered in the calculation.

3.7 MULTIVARIATE STATISTICAL ANALYSIS

3.7.1 Principal Components Analysis

Principal components analysis (PCA) is aimed at finding and interpreting hidden complex, and possibly causally determined, relationships between features in a dataset. Correlating features are converted to the so-called scores which are themselves noncorrelated (Einax *et al.*, 1997).

PCA modelling, *i.e.*, the approximation of a matrix by a model, defined by variables and a relatively small number of outer vector products, shows the correlation

structure of a data matrix $\boldsymbol{X}$, approximating it by a matrix product of lower dimension ($\boldsymbol{T}$x$\boldsymbol{P}^T$), called the principal components, plus a matrix of residuals ($\boldsymbol{E}$).

$$\boldsymbol{X} = 1x\overline{\boldsymbol{X}} + TxP^T + E \quad (3.10)$$

Where, the term $1x\overline{\boldsymbol{X}}$ represents the autoscaled variable averages matrix. The second term, the matrix product ($\boldsymbol{T}$x$\boldsymbol{P}^T$), models the structure, and the third term, the residual matrix $\boldsymbol{E}$, is a matrix of residuals, and contains the deviations between the original values and the projections, *i.e.*, contains the noise. $\boldsymbol{T}$ is a matrix of scores that summarizes the X-variables, and $\boldsymbol{P}$ is a matrix of loadings showing the influence of the variables on each score. Geometrically, it corresponds to fitting a line, plane or hyper plane to the data in the multidimensional space, with the variables as axes. The scaling of the variables specifies the length of the axes of this space

SIMCA-P (*Umetrics AB*) software package was used to perform the PCA. It iteratively computes one principal component at a time, comprising a score vector t_a and a loading vector p_a. The score vectors contain information on how the samples relate to each other. Otherwise, the loading vectors define the reduced dimension space and contain information on how the variables relate to each other. Usually, a few principal components (2 or 3) can express most of the variability in the data set when there is a high degree of correlation among data.

3.7.2 Mathematical Background

A dataset can be seen as a matrix of i samples and j variables summarising the main results of an experience. In Figure 3.7 is schematized the PCA process.

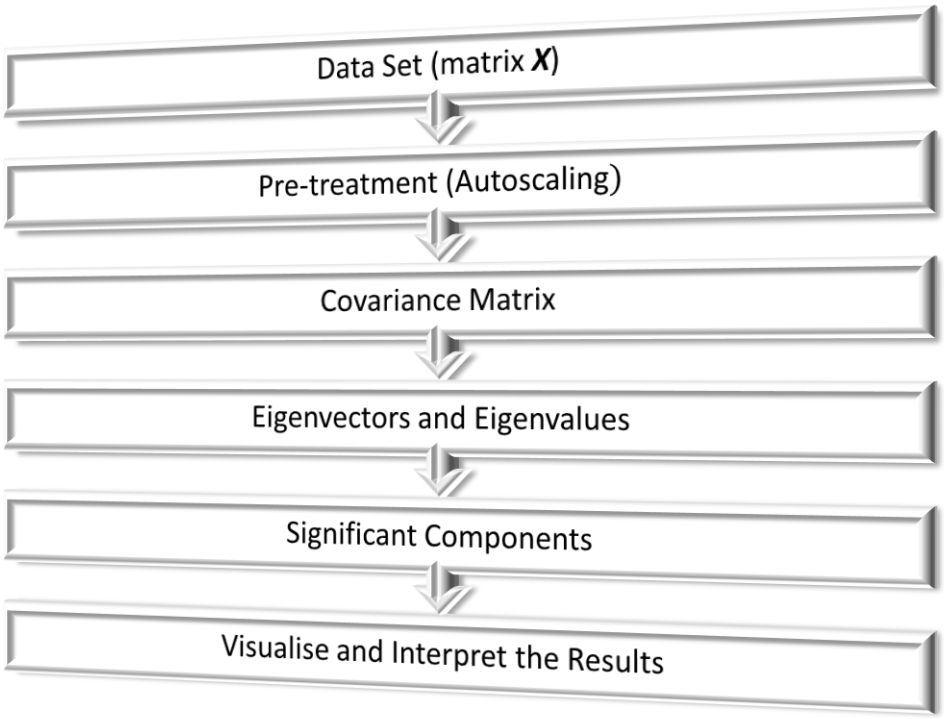


Figure 3.7 – Principal Components Analysis steps.

The first step consists in the pre-treatment of data by standardization of the variables, *i.e.*, guarantee that each individual variable has about the same range, avoiding that some variables would be more important than others because of scale effects. During this work each variable was autoscaled by:

$$z_{ij} = \frac{x_{ij} - \bar{x}_j}{s_j} \tag{3.11}$$

Where, x_{ij} is the value of the variable j in the sample i, $\bar{x}_j$ and s_j are the mean and the standard deviation of the variable j, respectively, and, z_{ij} is the autoscaled value of x_{ij}. At the end of this standardization, each variable has mean zero and unit standard deviation.

Afterwards, the covariance matrix is determined. This indicates how much the variables vary from the mean with respect to each other. The covariance matrix is given by:

$$\boldsymbol{C}^{nxn} = \left(c_{ij}, c_{ij} = cov\left(Dim_i, Dim_j\right)\right) \tag{3.12}$$

Where, $\boldsymbol{C}^{nxn}$ is a matrix with n rows and n columns, and Dim_x is the x^{th} dimension. This means that if we have an n-dimensional dataset, then the matrix has n-rows and columns (so is square) and each entry in the matrix is the result of calculating the covariance between two separate dimensions. If the covariation value between two variables is positive, it indicates that both dimensions increase together, and, if the value is negative, then as one dimension increases, the other decreases. If the covariance is zero, it indicates that the two dimensions are independent of each other.

Since the covariance matrix is square, we can calculate the eigenvectors and eigenvalues for this matrix. These are rather important, as they tell us useful information about our data. They are determined by:

$$\boldsymbol{C} \cdot \boldsymbol{e_1} = \lambda_1 \cdot \boldsymbol{e_1}$$
$$\boldsymbol{C} \cdot \boldsymbol{e_2} = \lambda_2 \cdot \boldsymbol{e_2} \tag{3.13}$$
...

$$|\boldsymbol{C} - \lambda \boldsymbol{I}| = 0 \tag{3.14}$$

Where, $\boldsymbol{C}$ is the covariance matrix, $\boldsymbol{e}$ is an eigenvector, and λ is an eigenvalue.

These mathematical measures show some essential properties (Einax *et al.*, 1997):

- Eigenvalues are a measure of the extracted variance from the total variable variance s^2_{total}, expressed by the correlation matrix $\boldsymbol{C}$;
- Eigenvalues are arranged in descending order:
  $\lambda_1 > \lambda_2 > \lambda_3 > \ldots > \lambda_n$ (3.15)
- The sum of all eigenvalues is equal to the number of variables n;
- Eigenvalues and eigenvectors are coupled pairs;
- Eigenvalues are orthogonal to each other.

The eigenvector with the highest eigenvalue is the first principal component of the dataset, showing the most significant relationship between the data dimensions. The second eigenvector, give other pattern in data, the second most important, and so on. The eigenvectors were normalized dividing by the square root of λ_j; they then have variance equal to unity and we get the matrix of factor loadings $\boldsymbol{P}$. These loadings are the weights of the original variables in the new variables (scores). Therefore the new variables are noncorrelated with each other, they have themselves the variance of one and contain a certain part of the total variance of the dataset expressed by their eigenvalues.

The criterion used to determine the model dimensionality (number of significant components) was cross validation (CV). Part of data is kept out of the model development, and then are predicted by the model and compared with the actual values. The prediction error sum of squares (PRESS) is the squared differences between observed and predicted values for the data kept out of the model fitting. This procedure is repeated several times until all elements have been kept out once and only once. Therefore, the final PRESS has contributions from all data. For every dimension, *SIMCA-P* computes the overall PRESS/SS, where SS is the residual sum of squares of the previous dimension. A component is considered significant if PRESS/SS is statistically smaller than 1.0.

PCA is a way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. Since patterns can be hard to find in data of high dimension, where graphical representation is not available, PCA is a powerful tool to visualise multidimensional data.

To represent observations in the space of factors is necessary to calculate the matrix of factor scores $\boldsymbol{T}$. The procedure is a multiple regression between the original values and the factors (principal components). The graphical representation of the observations may be used to detect groups, trends, and/or outliers.

The new variables (latent variables) are weighted sum of the original variables, x_j, as seen in the following equation (Massart and Heyden, 2004):

$$S_{i,\mathrm{PC1}} = \sum\left(w_{j,\mathrm{PC1}} \cdot z_{ij}\right) \tag{3.16}$$

Where, $S_{i,\mathrm{PC1}}$is the score of observation i on PC1, $w_{j,\mathrm{PC1}}$ is the loading of variable j on PC1, and z_{ij} is the autoscaled value of variable j in observation i.

Therefore, variables with high loadings (positive or negative value) on a certain score have strong influence in that score.

3.8 REFERENCES

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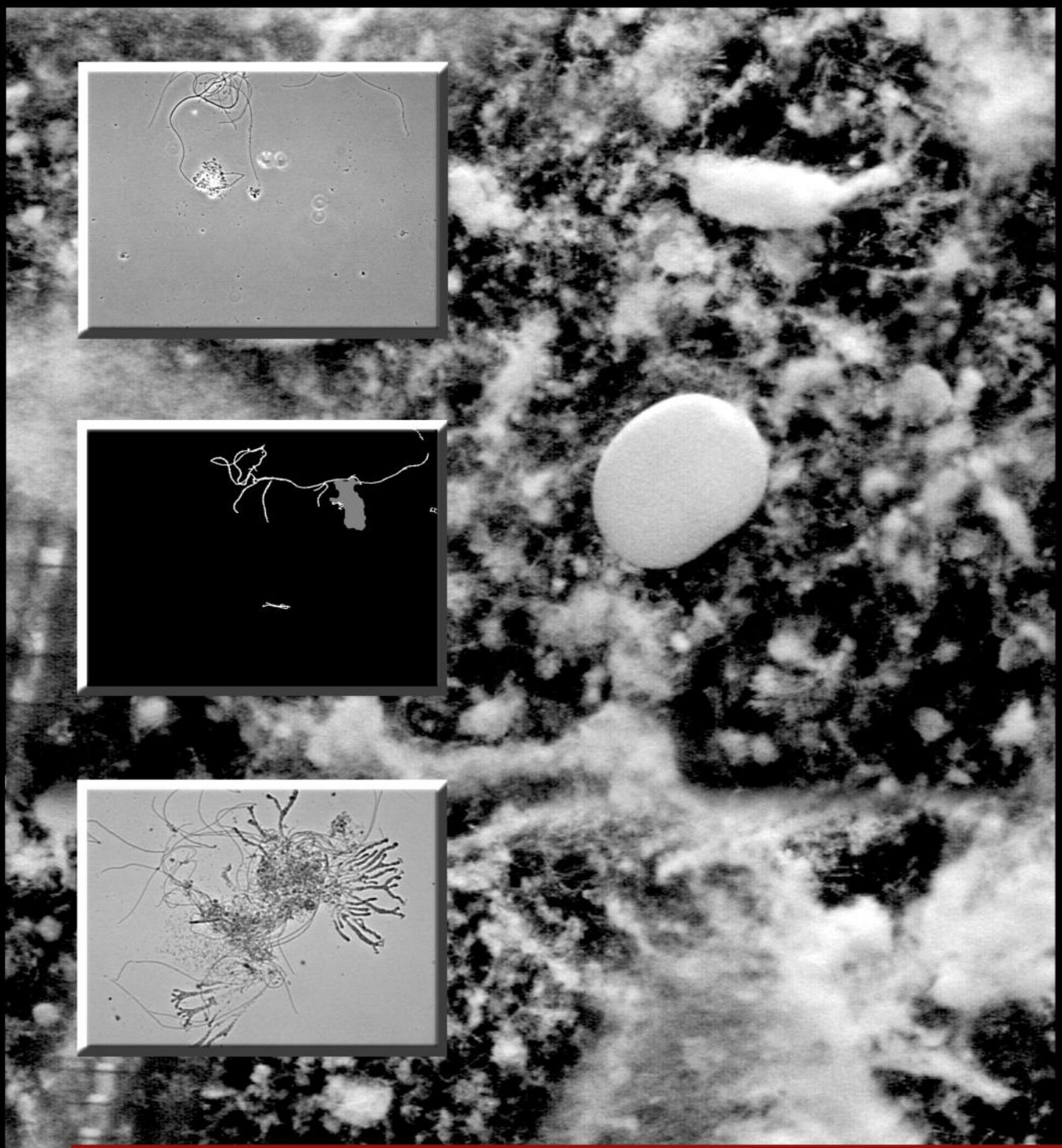
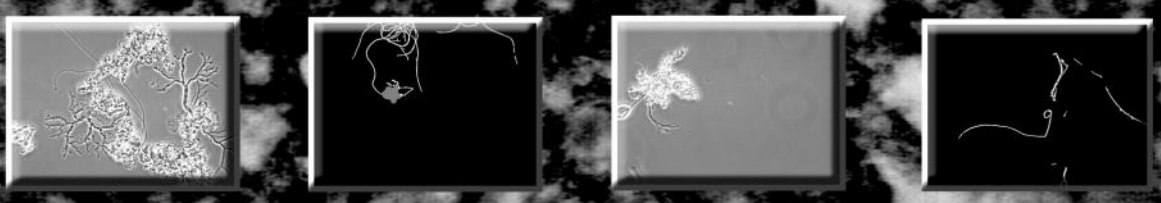
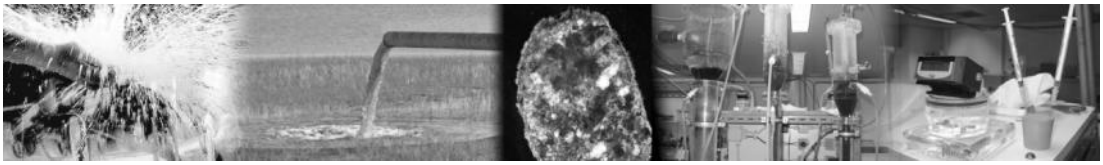


1 2 3 4 5 6 7 8

Organic Loading Disturbances





ABSTRACT

Four organic loading disturbances were performed in lab-scale EGSB reactors fed with ethanol. In load disturbance 1 (LD1) and 2 (LD2), the organic loading rate (OLR) was increased between 5 and 18.5 $kg_{COD}·m^{-3}·d^{-1}$, through the influent ethanol concentration increase, and the hydraulic retention time decrease from 7.8 to 2.5 h, respectively. Load Disturbances 3 (LD3) and 4 (LD4) were applied by increasing the OLR to 50 $kg_{COD}·m^{-3}·d^{-1}$ during 3 and 16 days, respectively. The sludge bed stability was quantified by image analysis and specific methanogenic activity assays and was related to the reactor performance, including effluent volatile suspended solids, indicator of washout events. Granules erosion/fragmentation was observed in LD1 since the % of aggregates projected area with D_{eq} > 1mm decreased from 90 to 70%. An immediate granules erosion/fragmentation was also observed in LD3 and LD4, quantified by a reduction of 45% in the % of projected area of aggregates with D_{eq} > 1mm. In general, it was observed the selective washout of filamentous forms associated to granules erosion/fragmentation and to a decrease in the specific acetoclastic activity. These phenomena induced the transitory deterioration of reactor performance in LD2, LD3 and LD4, but not in LD1. Extending the exposure time in LD4 promoted acetogenesis inhibition after 144h.

4.1 INTRODUCTION

Biological wastewater treatment plants are normally designed with reference to a nominal operating condition, in which the loading rate is assumed to be constant in time. However, in practice this steady-state assumption is seldom met and fluctuations, both in flow and influent concentration occur, which often result in performance degradation or even digester failure.

Anaerobic digesters run frequently at Organic Loading Rates (OLR) below the maximum process capacity. Stable operation of high-rate anaerobic processes is in practice, a difficult task. Due to the slow growth of syntrophic and methanogenic bacteria, anaerobic wastewater treatment technology is quite often unstable during influent fluctuations with volatile fatty acids accumulation and pH decrease, leading sometimes to process failure (Voolapalli and Stuckey, 1998). Depending on the monitoring plan and operator's expertise, the influent disturbances can exhibit different configurations: either as a short/transient overload, which only lasts few hours, or as a longer step change of days to weeks.

In the last decades, a significant number of experiments have been performed to study the effects caused by overloads in anaerobic digesters. Comparing an organic shock, at constant hydraulic retention time (HRT) and a hydraulic shock at constant influent chemical oxygen demand (COD), Nachaiyasit and Stuckey (1997) reported that the effect of reducing the HRT (with consequent increase of OLR) is more severe, and its effects are more complex. Cavaleiro et al (2001) studied the effect of hydraulic and organic shocks in an oleic acid fed reactor and concluded that the hydraulic shock promoted an increased tolerance of the sludge to oleic acid toxicity.

Fang and Yu (2000) studied the effects of HRT on mesophilic acidogenesis and concluded that the biodegradability of the major constituents in wastewater increased with HRT. Masse and Massé (2005) reported that hydraulic shock load had small and temporary effects on soluble COD and volatile fatty acids (VFA) concentrations, but effluent VSS concentrations increased.

The identification of relevant parameters for process monitoring is a key factor for efficient operation of the anaerobic wastewater treatment processes. Parameters involved in reactor control, have been limited to indicators of the liquid and the gaseous phases and have mostly been limited to the assessment of operational performance measurements. The impact on microbial community structure has rarely been investigated (McMahon et al. 2004), although the combination of physical and biological methods for process monitoring and control, has been proposed as a way to more effectively minimize problems of process imbalance in anaerobic digesters during load disturbance events (Voopalli and Stuckey, 1998). There are evidences that microbial and structural changes of biological aggregates can be detected before the detection of significant process performance deterioration after a disturbance (Amaral et al., 2004, Abreu et al., 2007).

The link between digester performance and the physiological and structural characteristics of the anaerobic sludge is particularly relevant in Up-flow Anaerobic Sludge Blanket (UASB), Expanded Granular Sludge Bed (EGSB) or Internal Circulation (IC) reactors (McMahon et al., 2004). Notwithstanding, limited knowledge about physiological and morphological changes of anaerobic sludge under unsteady state operating conditions is available in the literature. Considering that more that 2000 industrial applications of UASB/EGSB reactors are nowadays under operation all around the world, it is important to assess and provide quantitative data about granular sludge structure and physiological performance under a variety of normal and unstable conditions.

The use of quantitative image analysis techniques for process monitoring was previously applied in the quantification of morphological structure of anaerobic sludge under organic/hydraulic shock conditions using long chain fatty acids as substrate (Alves et al., 2000). Recently, Li et al. (2007) compared several characteristics of activated sludge flocs and granules by using different techniques including image analysis. The statistical tool Principal Component Analysis was used to relate image analysis information with the sludge settleability in terms of SVI (Jenné et al., 2006). The first attempts to use digital image analysis in high-rate anaerobic digestion processes were limited to size measurements and number counting (Dudley et al., 1993; Jeison and Chamy, 1998). Several subsequent works demonstrated the application of quantitative image analysis tools to monitor granulation and granules deterioration processes (Araya-kroff et al., 2004, Amaral et al., 2004), to evaluate the effect of detergents in the structure and microbial activity of granular sludge (Costa et al., 2007) or in the detection of structural and microbial changes of granular sludge during a process of acetoclastic activity recovery (Abreu et al., 2007). Quantitative image analysis minimizes the subjectivity and time-consuming inherent to traditional image analysis techniques based in microscope observation and manual counting.

This work intends to present new information, supplied by the integration of operational, physiological and morphological data provided by quantitative image analysis techniques, about changes occurred in anaerobic granular sludge during fluctuations in influent concentration and hydraulic retention time. Concurrently, the effect of exposure time in two severe load disturbances of 3 and 16 days is studied.

4.2 EXPERIMENTAL SECTION

The operating parameters, specific methanogenic activity methodology, and image analysis technique used during these experiments are described in Chapter 3 (Methodology) of this thesis.

4.2.1 Inoculum and substrate

400 mL of granular sludge from a lab-scale EGSB reactor, treating a synthetic effluent with ethanol as sole organic carbon source, was used as the inoculum of the four EGSB reactors used in these experiments. The biomass was characterized in terms of specific methanogenic activity (SMA) with acetate, and H_2/CO_2 as substrates, morphology by quantitative image analysis, and volatile suspended solids (VSS) (Table 4.1).

The reactors were fed with ethanol at a concentration of 1.5 $g_{COD}\cdot L^{-1}$. Sodium bicarbonate was added as the alkalinity source (3 $g\cdot L^{-1}$) and macro and micronutrients were added according to:

Macronutrients – $MgSO_4\cdot 7H_2O$: 30 $g\cdot L^{-1}$; KH_2PO_4: 28.3 $g\cdot L^{-1}$; NH_4Cl: 170 $g\cdot L^{-1}$. 0.6 mL of this solution was added per gram of COD fed.

Micronutrients – $FeCl_2\cdot 6H_2O$: 2$g\cdot L^{-1}$; H_3BO_3: 0.05 $g\cdot L^{-1}$; $ZnCl_2$: 0.05 $g\cdot L^{-1}$; $CuCl_2\cdot 2H_2O$: 0.038 $g\cdot L^{-1}$; $MnCl_2\cdot 4H_2O$: 0.5 $g\cdot L^{-1}$; $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$: 0.05 $g\cdot L^{-1}$; $AlCl_3\cdot 6H_2O$: 0.09 $g\cdot L^{-1}$; $CoCl_2\cdot 6H_2O$: 2 $g\cdot L^{-1}$; $NiCl_2\cdot 6H_2O$: 0.092 $g\cdot L^{-1}$; $Na_2SeO_3\cdot 5H_2O$: 0.164 $g\cdot L^{-1}$; EDTA: 1 $g\cdot L^{-1}$; Resazurin: 0.2 $g\cdot L^{-1}$; HCl 37%: 1 $mL\cdot L^{-1}$. The composition of this solution was based on the work of Zehnder *et al.* (1980) and was supplemented to the influent feed by addition of 1 ml per litre.

When the reactors were operating in steady-state, four organic load disturbances were applied (Table 4.1). The organic overload was performed by increasing the

substrate concentration (LD1, LD3 and LD4), and by decreasing the HRT (LD2). Load Disturbance 3 and 4 were performed by increasing the OLR to 50 $kg_{COD}·m^{-3}·d^{-1}$ during 3 and 16 days, respectively. The recovery phase was followed through 7 days.

Table 4.1 – Inocula characterization and load disturbances conditions

Load Disturbance	**1**	**2**	**3**	**4**
Inoculum Characterization:				
Specific Acetoclastic Activity ($mL_{CH4@STP}·g_{VSS}^{-1}·d^{-1}$)	234±15	367±20	234±15	328±12
Specific Hydrogenotrophic Methanogenic Activity ($mL_{CH4@STP}·g_{VSS}^{-1}·d^{-1}$)	1427±26	1686±52	1427±26	1720±44
Morphology				
LfA (mm^{-1})	20	18	20	18
TL/VSS ($m·g^{-1}$)	1099	1238	1099	1238
VSS/TA ($g·m^{-2}$)	18.4	15.4	18.4	15.4
D_{eq} macro-aggregates (mm)	1.06±0.58	0.91±0.67	1.06±0.58	0.91±0.67
VSS ($g·L^{-1}$)	14.78	33.57	14.78	33.57
Shock Load Conditions:				
Ethanol ($g_{COD}·L^{-1}$)	5	1.6	15	15
HRT (h)	8	2.5	8	8
OLR ($Kg_{COD}·m^{-3}·d^{-1}$)	15	15	50	50
Exposure Time (hours)	72	72	72	384
Recovery Phase (days)	7	7	7	7

4.3 RESULTS AND DISCUSSION

4.3.1 Reactor Performance

Four load disturbances (LD) were applied when the Expanded Granular Sludge Bed (EGSB) reactors were operating in steady state with organic loading rates (OLR) of roughly 5 $kg_{COD}·m^{-3}·d^{-1}$ and hydraulic retention time (HRT) of 8 h. The COD removal efficiencies were higher than 85 % (Figure 4.1). In LD1 and LD2 the organic loading rates (OLR) were increased to 18.5 $kg_{COD}·m^{-3}·d^{-1}$, through the substrate (ethanol) concentration increase and the hydraulic retention time (HRT) decrease to 2.5 hours, respectively. The LD3 and LD4 were performed by increasing the substrate concentration (OLR of 50 $kg_{COD}·m^{-3}·d^{-1}$) during 3 and 16 days, respectively.

The load disturbance LD1 did not cause deterioration in reactor performance. After a temporary decrease, the COD removal efficiency increased to more than 95 %, during LD1, suggesting that the reactor was operating, in the pre-disturbance conditions, far from its maximum capacity. In contrast, when the flow rate increased in LD2, the COD removal efficiency decreased and stabilized around 73 %, until the end of the disturbance. This led to the accumulation of acetate in the effluent that peaked to values of 300 $mg_{COD}·L^{-1}$ (data not shown).

When the OLR increased to 50 $kg_{COD}·m^{-3}·d^{-1}$ (LD3 and LD4), the COD removal efficiency decreased linearly, from 90 to 30 % (Figure 4.1), in the first forty hours of disturbance. After this period, and extending the exposure time (LD4), the COD removal efficiency was recurrently around 35 %. Few hours after the end of the shock exposure period, all reactors regained the pre-disturbance efficiencies (Figure 4.1).

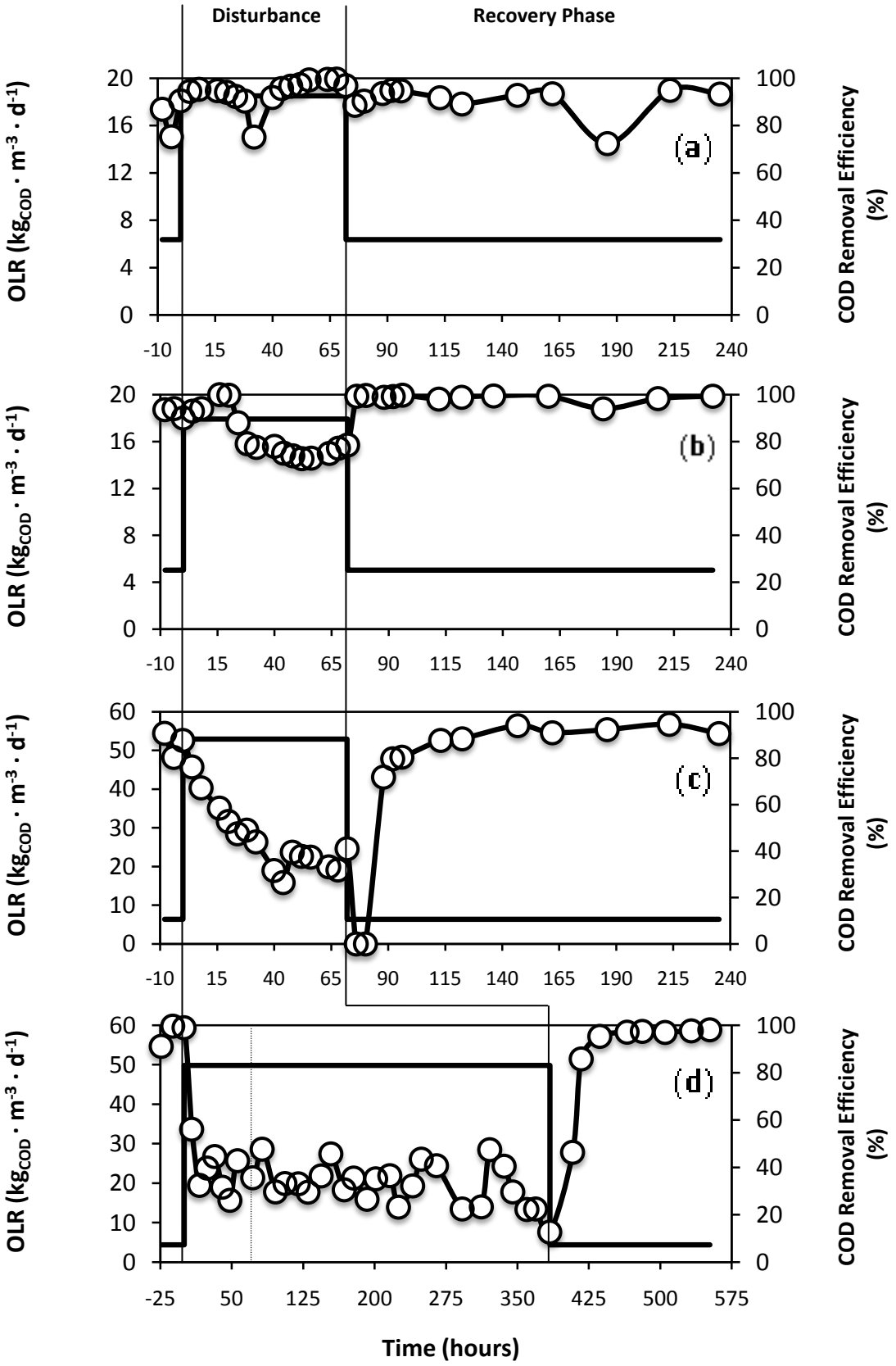


Figure 4.1 – Time course of Organic Loading Rate (OLR, ▬), and COD removal Efficiency (⊸), during LD1 (**a**), LD2 (**b**), LD3 (**c**), and LD4 (**d**).

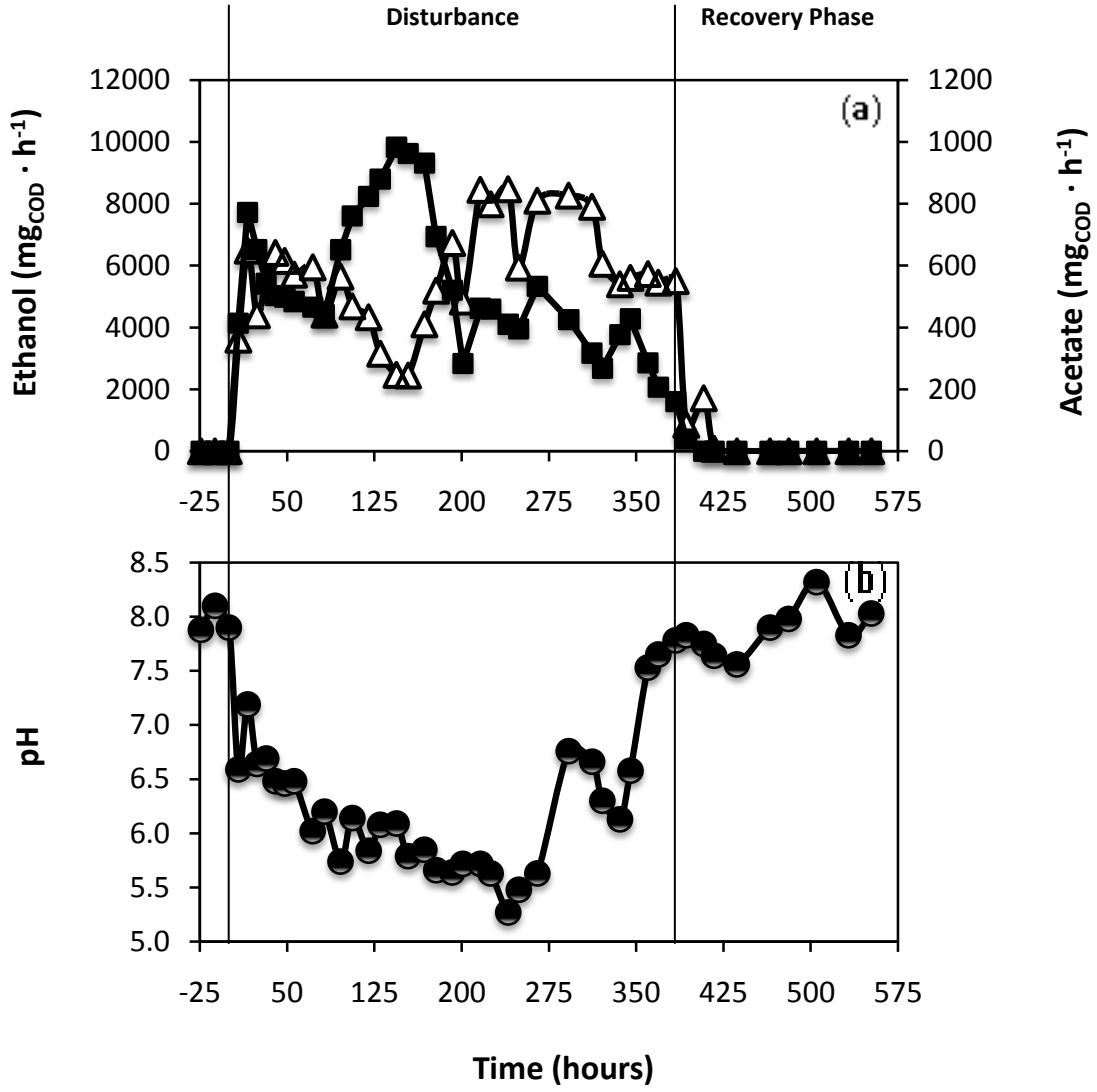


Figure 4.2 – Time course, during LD4, of: (**a**) ethanol (▪▪△▪▪) and acetate (▬■▬) concentration in the effluent; and, (**b**) pH (▬●▬).

With the deterioration of reactor capability to degrade the substrate available and consequent efficiency decrease, accumulation of acetate was observed in LD3 and LD4, achieving temporary peaks of 1400 (LD3, not shown) and 1000 $mg_{CODacetate} \cdot L^{-1}$ (Figure 4.2a), respectively. The conversion of ethanol via propionate as an intermediate occurred in this work as reported before by others (Laanbroek et al.,

1982; Abreu et al., 2007). Effluent propionate concentration increased to more than 70 $mg_{CODpropionate} \cdot h^{-1}$, in LD3 and LD4, immediately after the beginning of the overloads and decreased afterwards to residual values (data not shown). A fast decrease of pH from 7.5 to less than 6 was observed in the first hours of disturbances (Figure 4.2b). In LD3, the pH remained near 6, during all the period of shock exposure (data not shown), whereas in LD4, a gradual increase in the pH from 5.8 to 7 was observed in the last 150 hours of exposure (Figure 4.2b). The pH increase was simultaneous with a decrease in acetate concentration and an increase in the ethanol concentration up to 5000 $mg_{CODethanol} \cdot L^{-1}$ in LD3 (not shown) and 9000 $mg_{CODethanol} \cdot L^{-1}$ in LD4 (Figure 4.2), suggesting an inhibition of acetogenic reaction between ethanol and acetate.

4.3.3 Morphological, structural and physiological data

Quantitative morphological information retrieved by image analysis techniques can be related with the operational and physiological information in order to interpret the impact of load disturbances in the structure of granular sludge and consequently its impact in the sludge bed stability.

In the organic shocks imposed by increasing the influent concentration (LD1, LD3 and LD4) it was clearly observed a decrease in aggregates size. The percentage of aggregates projected area with equivalent diameter (D_{eq}) ≥ 1 mm decreased to 70 % in LD1, and to 58 % in LD3 and LD4. Concurrently, the % of projected area of aggregates inside the range of D_{eq} 0.1–1 mm increased to 28 and 40 %, respectively in LD1 (Figure 4.3a) and LD3/4 (Figure 4.3c,d). A higher flow of biogas production through the granular structure, induced by the increase in the substrate concentration, is likely responsible for the observed fragmentation, being expectable that the respective impact could be dependent on the magnitude of the load disturbance. In the hydraulic shock (LD2), a negligible change in size distribution was observed (Figure 4.3b). However, in this experiment, an increase in

the total filaments length per volatile suspended solids (TL/VSS) was observed immediately (8 h) after starting the disturbance period (Figure 4.4b), likely induced by the increasing shear stress inside the reactor, due to the higher up-flow velocity applied. In LD1 a negligible increase of free/protruding filaments was observed (Figure 4.4a), and in LD3/4 a peak of filaments occurred only 24 hours after increasing the load (Figure 4.4c,d).

It is interesting to observe that, although the granules were fragmented, LD1 did not impact the reactor performance (Figure 4.1a), but LD2 affected the reactor efficiency (Figure 4.1b), likely due to the erosion of filamentous bacteria from the granules, followed by detachment and washout. Therefore, by evaluating reactor's performance, it can be concluded that stability in the granules size distribution is of minor importance when compared to the capacity of filaments retention in the granular microbial structures.

Reactor performance deterioration was also subsequent to a high release of filaments in LD3 and LD4. Although granular sludge is a complex structure, it is accepted that, in general, acetoclastic bacteria are located mainly in the core of a stratified microbial structure (Harmsen et al. 1996, Liu et al., 2002a). This fact allows them to be more protected against operational problems such as overloads, pH oscillations or presence of toxic compounds (Liu et al., 2002b). In result of the observed fragmentation, the inner core of the granules becomes exposed to the bulk conditions, promoting the methanogenic archaea release and selective washout. Because filamentous forms increased in the bulk, in LD2, LD3 and LD4 (Figure 4.4b,c,d), it is worth to suggest that the filamentous forms could have been selectively washed out during those load disturbances, causing a decrease in the COD removal efficiency (Figure 4.1b,c,d). The observed decrease in the specific acetoclastic activity (SAA), especially during LD3 and LD4 (Figure 4.5c,d), the increase of effluent VSS (Figure 4.6b,c,d), and the kinetic characteristics of the *Methanosaeta* genera, which is a very slow growing archaea, may allow to speculate that *Methanosaeta* genera could have been selectively washed out during

LD2, LD3 and LD4. Apparently, extending the exposure time in LD4, the deterioration of reactor performance was persistent and simultaneous with the increase in effluent VSS concentration. This was likely due to filaments release, detachment and washout, inducing minimal values, both for filaments (Figure 4.4d) and SAA (Figure 4.5d).

It was observed in different previous works (Abreu et al. 2007, Araya-Kroff et al. 2004) that aggregation and densification of aggregates was simultaneous with an increase in the SHMA. In this respect, it is remarkable that, particularly during the recovery phase, the SHMA (Figure 4.5) and the VSS/TA (Figure 4.4) presented similar trends in almost all analysis.

The LfA parameter quantifies the level of biomass aggregation by measuring the ratio between the total filaments length and the projected area of the aggregates. A high correlation with the total filaments length per VSS was observed. Amaral (2003) and Costa et al. (2007) proposed that LfA could be an early alert of washout during influent disturbances in EGSB reactors, particularly when toxic substances were accidentally fed. However, in the present study, the effects on the morphology caused by the overloads were simultaneous with the increase in the effluent VSS. Therefore, the LfA parameter followed the trend of effluent VSS without advance (Figure 4.6), forbidding an earlier detection of the washout phenomenon.

By the end of the exposure periods, the turbulence/instability caused by the raise in biogas production inside the reactors, decreased. The effluent VSS decreased to values similar to the pre-disturbance conditions (Figure 4.6). During the recovery phase the morphological parameters stabilized in values close to the initial ones (Figure 4.4 and Figure 4.6), with the exception of aggregates size distribution (Figure 4.3).

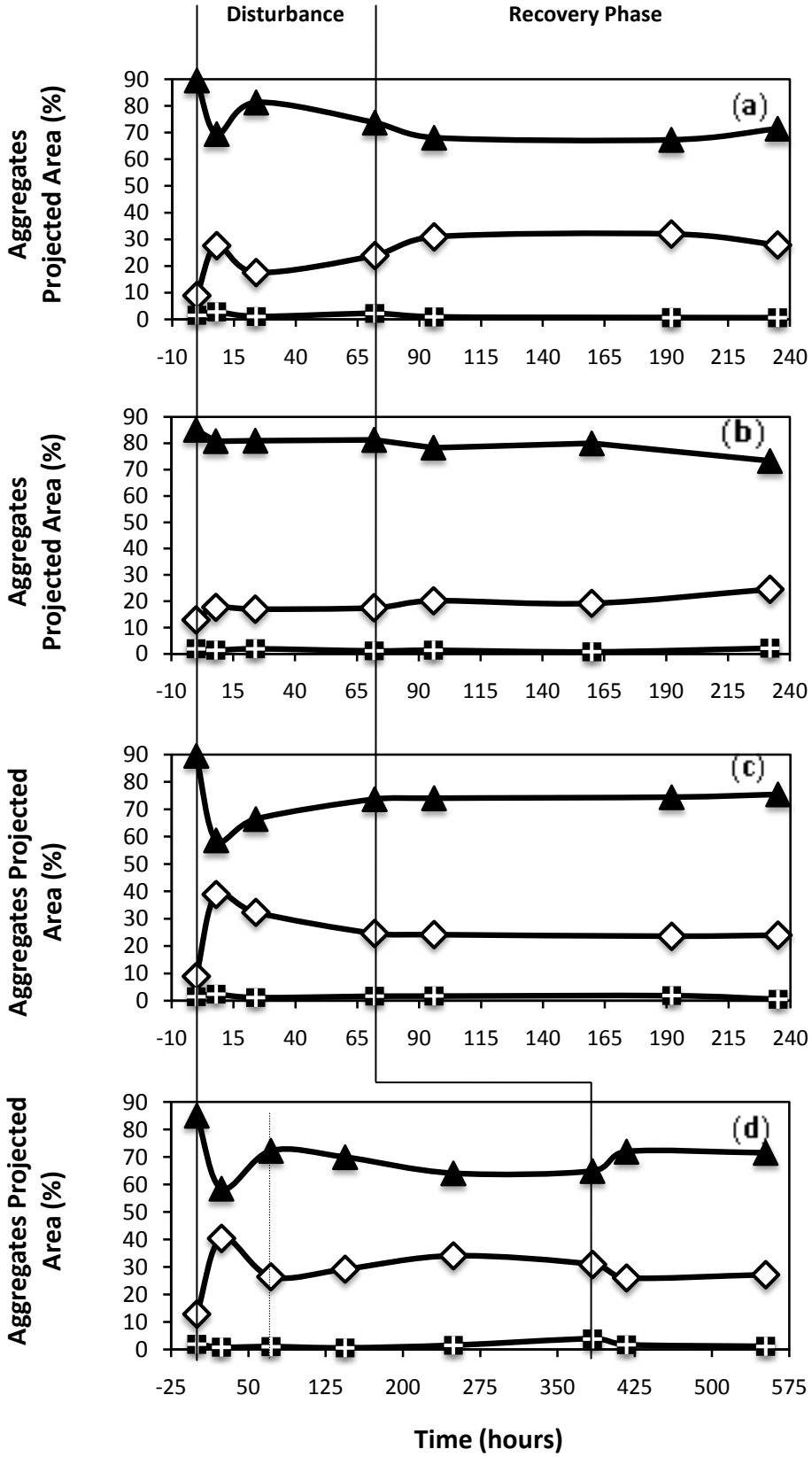


Figure 4.3 – Time course of the percentage of aggregates projected area by equivalent diameter (D_{eq}) ranges: D_{eq}≥1mm; 0.1mm≤D_{eq}<1mm; D_{eq}<0.1mm; during LD1 (**a**), LD2 (**b**), LD3 (**c**), and LD4 (**d**).

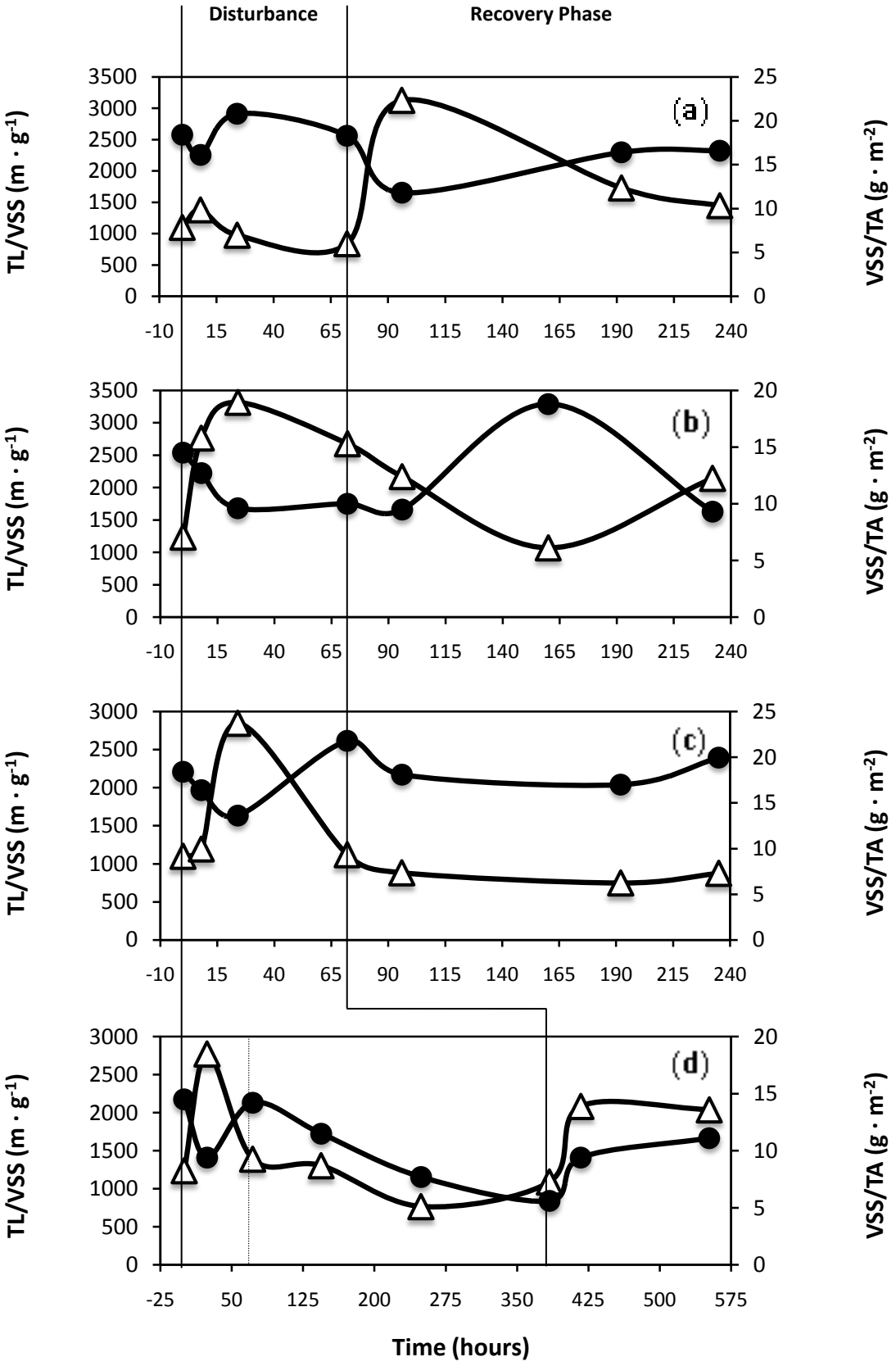


Figure 4.4 – Time course of the total filament length per volatile suspended solids (TL/VSS, —△—), and volatile suspended solids per total aggregates projected area (VSS/TA, ▪—●—▪), during LD1 (**a**), LD2 (**b**), LD3 (**c**), and LD4 (**d**).

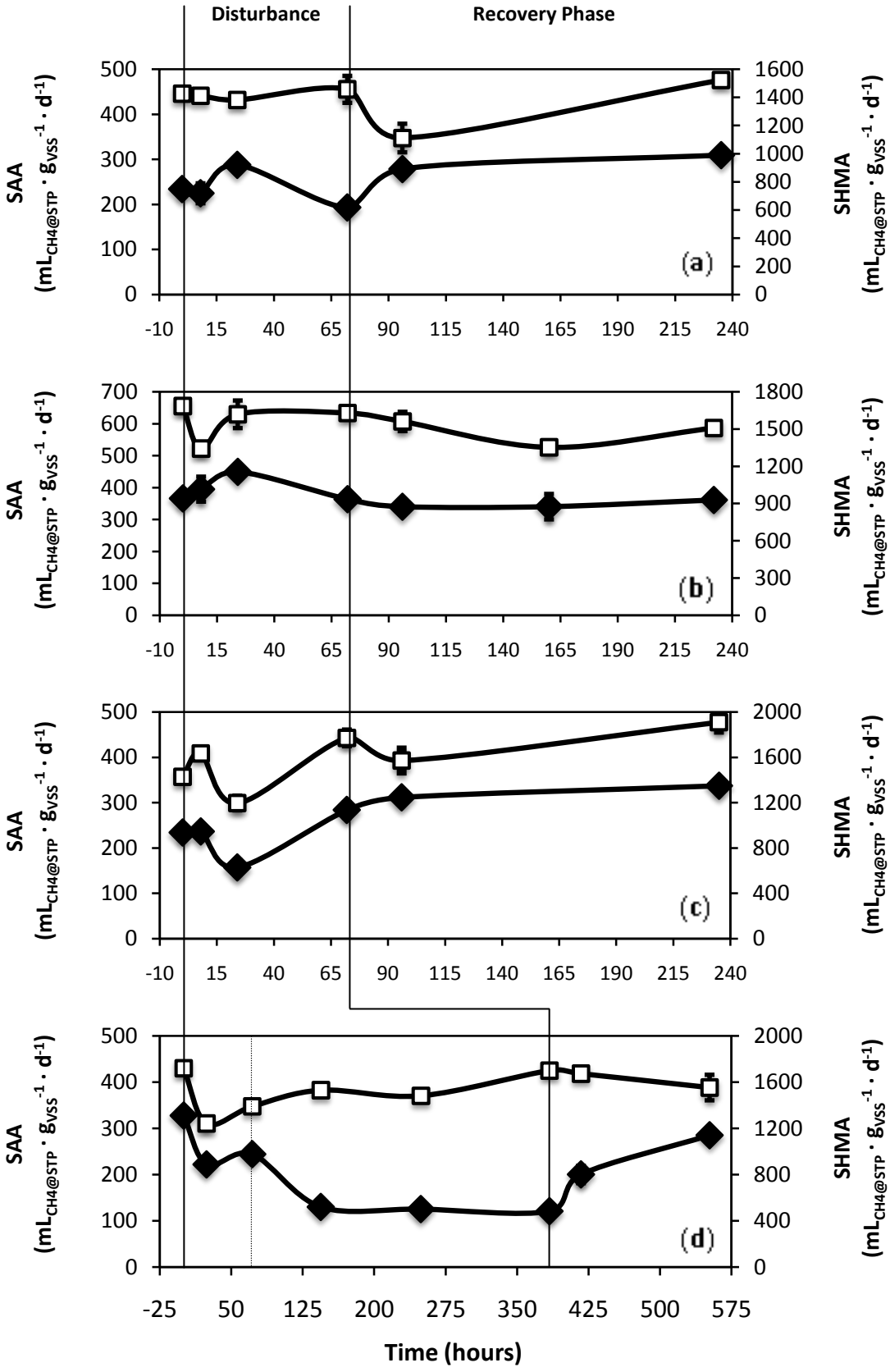


Figure 4.5 – Time course of specific acetoclastic activity (SAA, ◆), and specific hydrogenotrophic methanogenic activity (SHMA, □), during LD1 (**a**), LD2 (**b**), LD3 (**c**), and LD4 (**d**).

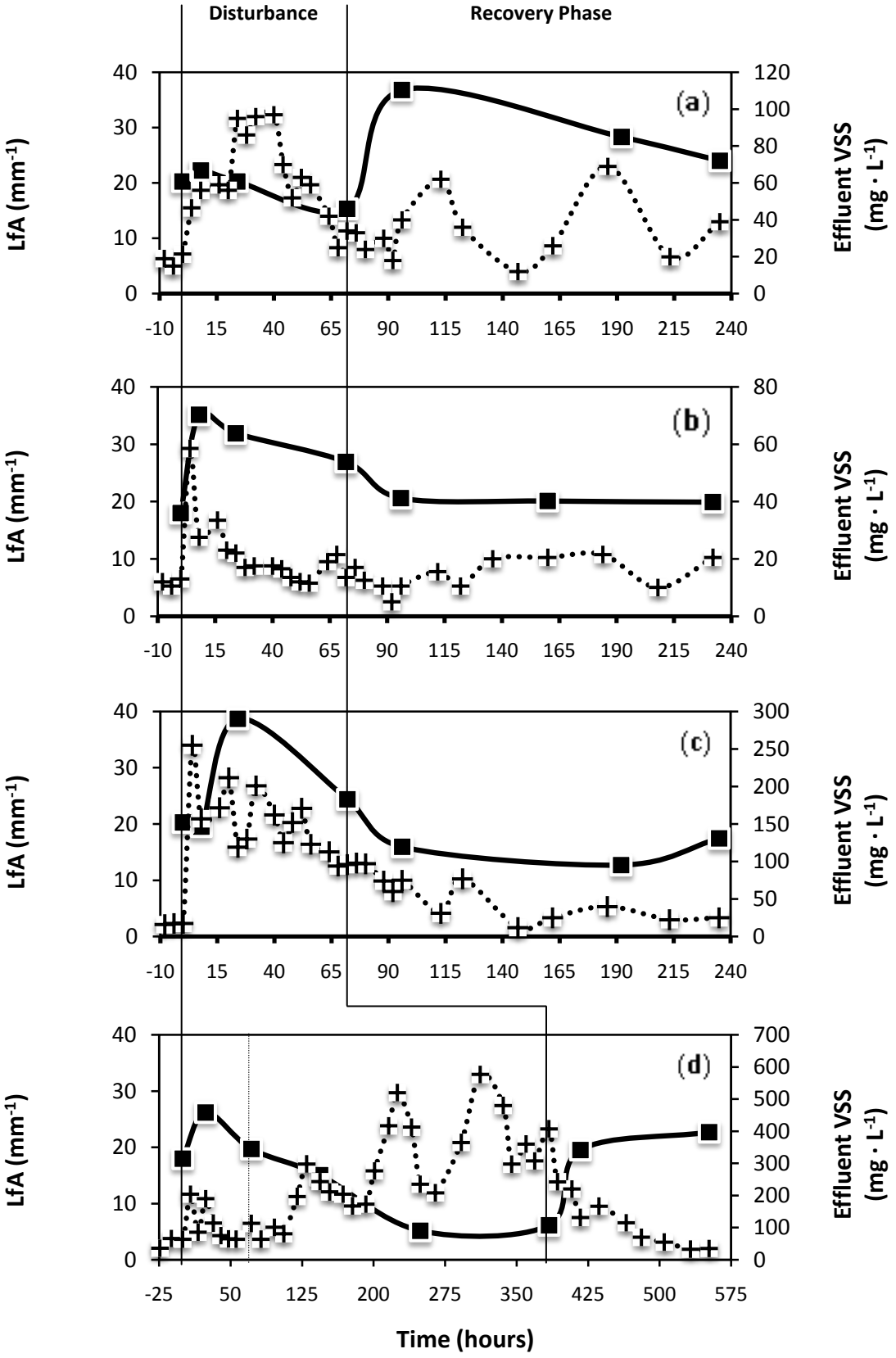


Figure 4.6 – Time course of the dynamic between total filament length and total aggregates projected area (LfA, ▬■▬), and, effluent volatile suspended solids (effluent VSS, • •+• •), during LD1 (**a**), LD2 (**b**), LD3 (**c**), and LD4 (**d**).

4.4 CONCLUSIONS

The effects of organic load disturbances on the operational, physiological and morphological properties of anaerobic granular sludge were studied by combining quantitative image analysis, methanogenic activity assays and performance data.

No significant effects were detected in the reactor performance during LD1 (OLR increased to 18 $kg_{COD}·m^{-3}·d^{-1}$). The COD removal efficiency was enhanced showing that the reactor was not operating at its maximum capability. During LD2 (HRT decrease to 2.5h), the reactor efficiency dropped from 94 to 72 % in the last hours of shock load. LD3 (OLR of 53 $kg_{COD}·m^{-3}·d^{-1}$, during 3 days) and LD4 (OLR of 50 $kg_{COD}·m^{-3}·d^{-1}$, during 16 days) caused the decrease of COD removal efficiency, from 90 to less than 30%, and, the accumulation of Volatile Fatty Acids caused a drop in the pH from 7.5 to less than 6. Increasing the exposure time (LD4) caused the pH raise coupled with the decrease in acetate and increase of ethanol concentration in the bulk, suggesting acetogenic inhibition.

In the organic shocks imposed by increasing the influent concentration (LD1, LD3 and LD4) granules fragmentation was observed. However, in hydraulic shock (LD2), a negligible change in size distribution, caused by erosion, was observed although an increase in TL/VSS was detected. Therefore, by evaluating reactor's performance, it was concluded that stability in the granules size distribution is of minor importance when compared to the capacity of filaments retention in the granular microbial structures.

The entire reactor's performance, physiological and morphological parameters regain its initial values few hours after the load disturbances stopped, with the exception of granules size distribution.

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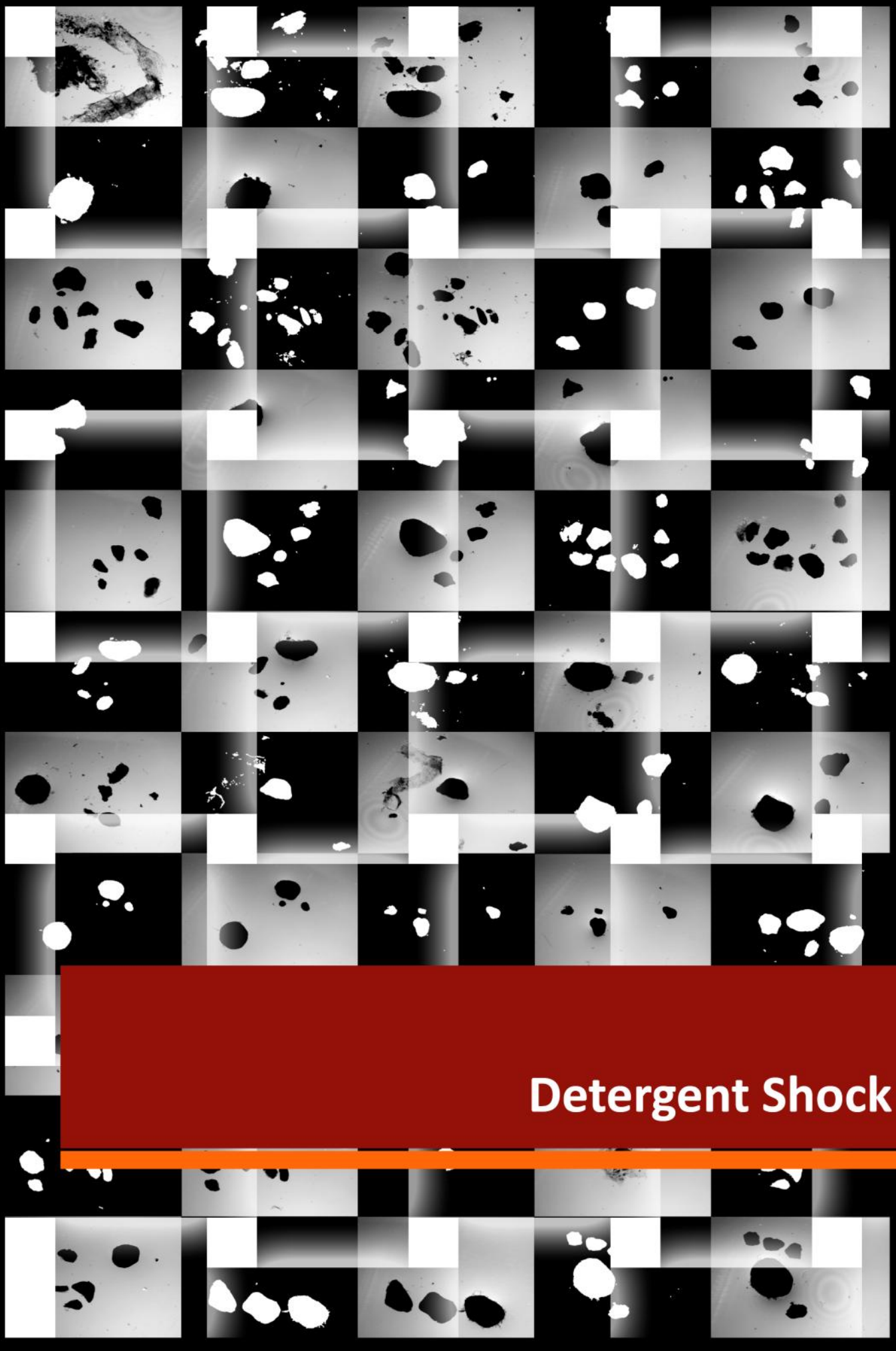
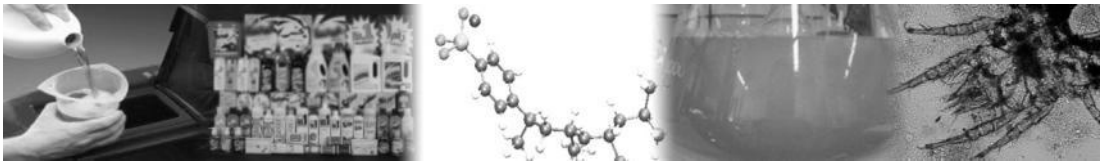


1 2 3 4 5 6 7 8

Detergent Shock Load



ABSTRACT

Two shock loads of a commercial detergent (I - 150 $mg_{COD}·L^{-1}$, fed for 56 h; II - 300 $mg_{COD}·L^{-1}$ fed for 222 h) were applied in a lab-scale EGSB reactor, fed with 1.5 $g_{COD}·L^{-1}$ of ethanol. The impact of the surfactant was assessed in terms of granular sludge morphology, specific methanogenic activity (SMA) in the presence of individual substrates, and reactor performance. COD removal efficiency remained unaffected in the shock I, but 80 h after starting exposure to shock II, the COD removal efficiency decreased drastically from 75 to 17%. In the first 8 h of operation of shock I, the SMA was stimulated and decreased afterwards, being recovered 5 days after the end of exposure time. Concerning to shock II, the SMA was immediately and persistently reduced during the exposure time, although, the inhibition of Specific Hydrogenotrophic Methanogenic Activity showed a trend to increase after the exposure time. Acetoclastic bacteria were observed as the most sensitive to the toxic effects of surfactant whereas the hydrogenotrophic bacteria were less affected. The inhibitory effects were dependent on surfactant concentration and exposure time. The ratio filaments length per total aggregates area (LfA) was an early-warning indicator of biomass washout, since it increased 3 and 5 days before effluent volatile suspended solids rise, respectively in shock I and II.

5.1 INTRODUCTION

Industrial wastewaters that are treated by anaerobic technology or have a potential for anaerobic treatment are frequently affected by temporary surfactant loads from cleaning processes (Nagel *et al.*, 1999). In order to optimize the treatment of these wastewaters it is important to know the effects of surfactants on the activity and physical properties of anaerobic aggregates.

Surfactants are water-soluble surface-active agents comprised of a hydrophobic portion, usually a long alkyl chain, attached to hydrophilic functional groups. Detergents, in general, are a class of compounds known to negatively affect the performance of anaerobic digesters (Tanaka and Ichikawa, 1993; Gavala and Ahring, 2002, Feitkenhauer and Meyer, 2002). The active matter of detergents is composed of natural and/or synthetic surfactants of different types (Prats *et al.*, 1997).

Most of the detergents are removed by attachment to the suspended solids (Prats *et al.*, 1997) due to surfactants properties of adsorption (Mosche and Meyer, 2002) and self-assembly. Therefore, surfactant molecules are usually found at the interface between a liquid and a solid phase, influencing its macroscopic properties of wetting, foaming, detergency and emulsion formation. Self-assembly is the tendency for surfactant molecules to organise themselves into extended structures in water. These structures are formed when the hydrophobic tails of the surfactants cluster together, forming small aggregates such as micelles, or large layer structures (bi-layers) which are similar to a cell wall.

Linear Alkylbenzene Sulfonate (LAS) are the most commom synthetic anionic surfactants used as domestic and industrial detergent (Gavala and Ahring, 2002;

Ying, 2006) and are very difficult to be degraded under anaerobic conditions because of their high toxicity to acetoclastic methanogens (Wagner and Schink, 1987; García-Morales *et al.*, 2001). This inference was drawn based on the fact that inhibition of methanogenesis was accompanied by the accumulation of acetate (Shcherbakova *et al.*, 1999).

Studies on LAS toxicity indicates that inhibition increases with longer exposure time (Ferrer and Berna, 1999) what suggests that more factors might be influencing the extent of inhibition than just the concentration of surfactants (Mosche and Meyer, 2002).

Granular sludge morphology can be monitored by quantitative image analysis methodologies which allow understanding mechanisms of erosion, fragmentation, filaments release and growth. Image analysis was already used to describe the changes on morphology of activated sludge flocs exposed to surfactants (Liwarska-Bizukojc and Bizukojc, 2005), but no reports exist about the effect of such compounds on the structure and morphology of anaerobic granular sludge.

Quantitative image analysis techniques were already applied to the characterization of anaerobic granular sludge and provided objective information about the mechanisms of granulation and granules deterioration processes (Araya-Kroff *et al.*, 2004, Amaral *et al.*, 2004). A parameter relating the free (and protruding) filaments length and the total aggregates projected area was proposed to quantify the morphological structure of the aggregates. This indicator evidenced to be sensitive to alert, with some anticipation, a washout event in an Expanded Granular Sludge Blanket (EGSB) reactor (Amaral *et al.*, 2004).

Integration of morphological analysis of granular sludge, operational and physiological information is unquestionably a relevant method that allows predicting overall performance and stability of high rate anaerobic reactors in the presence of influent fluctuations, either in terms of flow rate or composition. Surfactants, which are important potential toxicants for anaerobic granular sludge

reactors, were never studied in this perspective. The present work aims at presenting and discussing experimental results focused on the effect of detergent shock loads on operational performance and physiological activity of EGSB reactors, combined with morphological information retrieved from quantitative image analysis of anaerobic granular sludge at micro and macro structure levels.

5.2 EXPERIMENTAL SECTION

The operating parameters, specific methanogenic activity methodology, and image analysis technique used during these experiments are described in Chapter 3 (Methodology) of this thesis.

5.2.1 Detergent characteristics

The industrial detergent tested presents a relative density at 20 ºC of 1.04, a pH (81% solution at 20 ºC) of 11.4 and Chemical Oxygen Demand (COD) of 98 $g \cdot L^{-1}$. It is composed by glycol ether (1-10 %), anionic surfactant (1-10 %), performance additives (1-10 %), dyes (<1 %) and water (>60 %).

5.2.2 Inoculum and substrate

400 mL of granular sludge from a lab-scale EGSB reactor treating a synthetic effluent, with ethanol as sole organic carbon source, was used as the inoculum of the EGSB reactor used in these experiments. The inoculum was characterized in terms of morphology, specific acetoclastic activity (SAA), specific hydrogenotrophic

methanogenic activity (SHMA), settling velocity, and volatile suspended solids (VSS) (Table 5.1).

Table 5.1 – Inocula characterization and detergent shock loads conditions.

SHOCK LOAD	I	II
Inoculum Characterization:		
Specific Acetoclastic Activity ($mL_{CH4@STP} \cdot g_{VSS}^{-1} \cdot d^{-1}$)	150 ± 22	141 ± 8
Specific Hydrogenotrophic Methanogenic Activity ($mL_{CH4@STP} \cdot g_{VSS}^{-1} \cdot d^{-1}$)	833 ± 136	1028 ± 103
Morphology		
LfA (mm^{-1})	30	18
TL/VSS ($m \cdot g^{-1}$)	1585	1238
VSS/TA ($g \cdot m^{-2}$)	19	27
Deq macro-aggregates (mm)	0.84 ± 0.49	0.91 ± 0.67
VSS ($g \cdot L^{-1}$)	22	34
Settling velocity ($m \cdot h^{-1}$)	29.7 ± 8.7	30.6 ± 10.2
Shock Load Conditions:		
Ethanol ($mg_{COD} \cdot L^{-1}$)	1500	1500
Detergent ($mg_{COD} \cdot L^{-1}$)	150	300
Exposure Time (h)	56	222
Recovery Phase (days)	14	12

Ethanol was fed at a concentration of 1.5 $g_{COD} \cdot L^{-1}$. Sodium bicarbonate was added as the alkalinity source (3 $g \cdot L^{-1}$) and macro and micronutrients were added according to:

Macronutrients – $MgSO_4 \cdot 7H_2O$: 30 $g \cdot L^{-1}$; KH_2PO_4: 28.3 $g \cdot L^{-1}$; NH_4Cl: 170 $g \cdot L^{-1}$. 0.6 mL of this solution was added per gram of COD fed.

Micronutrients – $FeCl_2 \cdot 6H_2O$: 2 $g \cdot L^{-1}$; H_3BO_3: 0.05 $g \cdot L^{-1}$; $ZnCl_2$: 0.05 $g \cdot L^{-1}$; $CuCl_2 \cdot 2H_2O$: 0.038 $g \cdot L^{-1}$; $MnCl_2 \cdot 4H_2O$: 0.5 $g \cdot L^{-1}$; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$: 0.05 $g \cdot L^{-1}$; $AlCl_3 \cdot 6H_2O$: 0.09 $g \cdot L^{-1}$; $CoCl_2 \cdot 6H_2O$: 2 $g \cdot L^{-1}$; $NiCl_2 \cdot 6H_2O$: 0.092 $g \cdot L^{-1}$; $Na_2SeO_3 \cdot 5H_2O$: 0.164 $g \cdot L^{-1}$; EDTA: 1 $g \cdot L^{-1}$; Resazurin: 0.2 $g \cdot L^{-1}$; HCl 37%: 1 $ml \cdot L^{-1}$. The composition of this solution was based on the work of Zehnder *et al.* (1980) and was supplemented to the influent feed by addition of 1 ml per litre.

When the reactors were operating in steady-state, two shock loads were then applied (Table 5.1). In the first shock load, a detergent concentration of 150 $mg_{COD} \cdot L^{-1}$ was used during 56 h and the recovery was followed through 14 days. In the second shock load the detergent concentration was 300 $mg_{COD} \cdot L^{-1}$, the exposure time was 222 h, and the recovery phase was monitored during 12 days.

5.3 RESULTS AND DISCUSSION

5.3.1 Shock Load with 150 $mg_{COD} \cdot L^{-1}$ of Detergent

5.3.1.1 Operational Parameters

The reactor was operated at a hydraulic retention time (HRT) of 7.8 h, and it was fed with 1650 $mg_{COD} \cdot L^{-1}$ during the exposure time. When the detergent was removed from the feeding (after 56 h), the COD was 1380 $mg_{COD} \cdot L^{-1}$, corresponding to a reduction on the organic loading rate (OLR) from 5.0 to 4.3 $kg_{COD} \cdot m^{-3} \cdot d^{-1}$ (Figure 5.1).

No significant effects were observed in the reactor performance caused by the shock load. The COD detected in the effluent was roughly constant during the exposure and recovery phases. Consequently the COD removal efficiency was

consistently above 80% throughout the operational period. The pH changed from 8.0 ± 0.1 during the exposure time to 7.8 ± 0.2 in the recovery phase. This fact suggests that the detergent concentration did not affect significantly the pH inside the reactor.

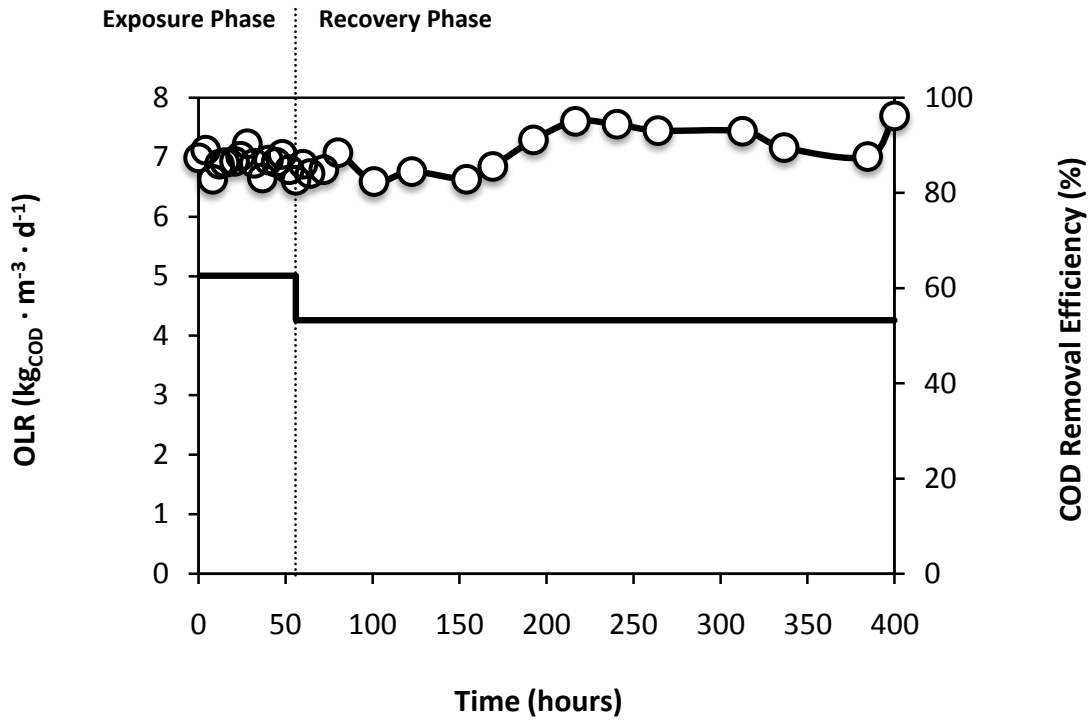


Figure 5.1 – Time course of operational parameters in shock load I: Organic Loading Rate (OLR, ▬), and COD removal Efficiency (⊸○⊸).

5.3.1.2 Methanogenic Activity

Figure 5.2, shows the Specific Acetoclastic Activity (SAA) and Specific Hydrogenotrophic Methanogenic Activity (SHMA), measured through the exposure and recovery phases.

At the beginning of shock load (first 8h) the Specific Methanogenic Activity (SMA) was stimulated by the detergent. It was already reported by Mosche and Meyer

(2002) that at low concentrations (< 3 $mg \cdot L^{-1}$), surfactants can induce stimulation on bacterial activity. However, after the initial stimulation, the SHMA was inhibited during the next 24 h of exposure and started to recover afterwards. The decrease in the SAA was more pronounced than in the SHMA, once it decreased until the end of the exposure period. The results suggest that the SAA might be the most sensitive to the toxic effects of the detergent. Other authors already reported that acetoclastic methanogens were more affected by surfactant toxicity (García-Morales *et al.*, 2001; Mosche and Meyer, 2002).

During the recovery phase the SMA achieved higher values than the ones presented by the inoculum. This suggests that the effects of detergent shock load with 150 $mg_{COD} \cdot L^{-1}$ of detergent were reversible approximately 113 hours after the end of exposure time and therefore were not permanent.

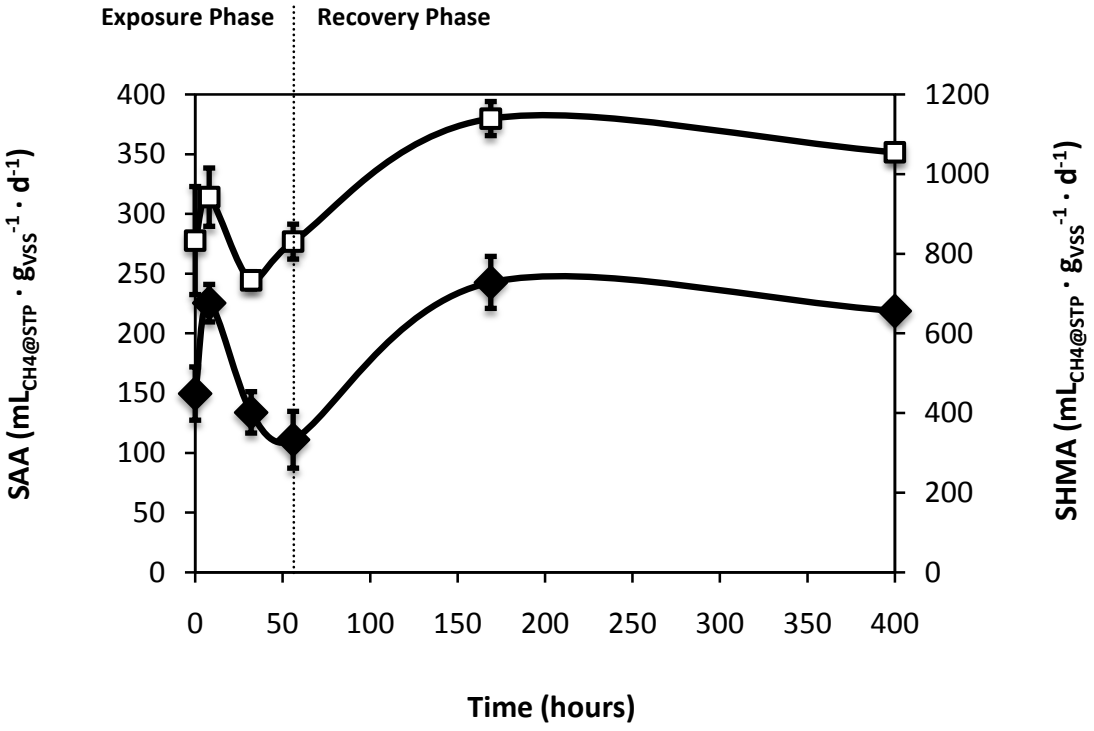


Figure 5.2 – Time course of the specific acetoclastic activity (SAA, ◆), and specific hydrogenotrophic methanogenic activity (SHMA, □), in shock load I.

5.3.1.3 Image Analysis

The use of image analysis techniques allowed the quantification of several changes in morphological parameters of biomass, such as, the ratio between total filaments length (free and protruding filaments) and total aggregates projected area (LfA). This parameter represents the dynamics involving filaments and aggregates inside the reactor. The total filaments length per volatile suspended solids (TL/VSS) and the percentage of total projected area of aggregates distribution in each equivalent diameter classes characterizes the filament and the aggregate dynamics individually. The ratio between volatile suspended solids and total projected area of aggregates (VSS/TA) reflects the apparent granules density.

During the first 8 h of exposure, the detergent toxic effects on anaerobic granular sludge were showed by an increase of filamentous forms released from the aggregates, represented by the TL/VSS increase (Figure 5.3b), that induced an increase of LfA value in the bulk (Figure 5.3a). In this first stage, the granules become less dense as evidenced by the VSS/TA decrease (Figure 5.3b). This might be explained by the surfactants adsorption and self-assembly properties. This fact was also verified by the decrease of granules average settling velocities (v_{sed}) from 29.7 ± 8.7 to 25.1 ± 10.8 $m \cdot h^{-1}$.

After this adaptation phase, and regardless of the intrinsic evolution of both parameters involved in the LfA definition, it was observed a significant increase of LfA until the end of exposure time (56 h), which was a result of the important release of filaments detected by the TL/VSS increase. The percentage of projected area of aggregates ≥ 1 mm was consistently above 80% throughout this period (Figure 5.3c) although an increase in the percentage of aggregates smaller than 0.1 mm was detected, possibly due to adsorption or self-assembly of surfactants onto the granules.

The last stage corresponds to the recovery phase. Here, LfA starts to decrease, suggesting a change in the dynamics of filaments and aggregates. This may be

explained by the washout of the free filaments and consequent TL/VSS decrease from 6150 to 870 $m \cdot g_{VSS}^{-1}$ (Figure 5.3b), and the enhancement in consistency of the granules as consequence the VSS/TA increased from 10 $g_{VSS} \cdot m^{-2}$ to 20 $g_{VSS} \cdot m^{-2}$. The v_{sed} slightly increased from 25.1 ± 10.8 to 28.2 ± 14.7 $m \cdot h^{-1}$. During this phase the effluent volatile suspended solids (VSS) started to increase (Figure 5.3a). LfA raised between 0 and 56 h and effluent VSS only increased after 101 h of operation, suggesting that LfA might be an earlier warning indicator of washout in detergent shock loads. Amaral *et al.* (2004) provides an explanation to the possible earlier detection of washout based on the mechanism of filaments release, detachment, selective washout, and LfA calculation method.

According to these authors, when the granules begin to fragment, the filaments start to be released from the granules into the bulk, consequently, the LfA increases due to detection of free filaments and filaments still attached to the granules (protruding filaments). Only in a subsequent phase, when the washout of the free filaments and small aggregates occurs, the effluent VSS increases. In this phase the LfA decreases, since inside the reactor only the bigger aggregates and the residual protruding filaments remain.

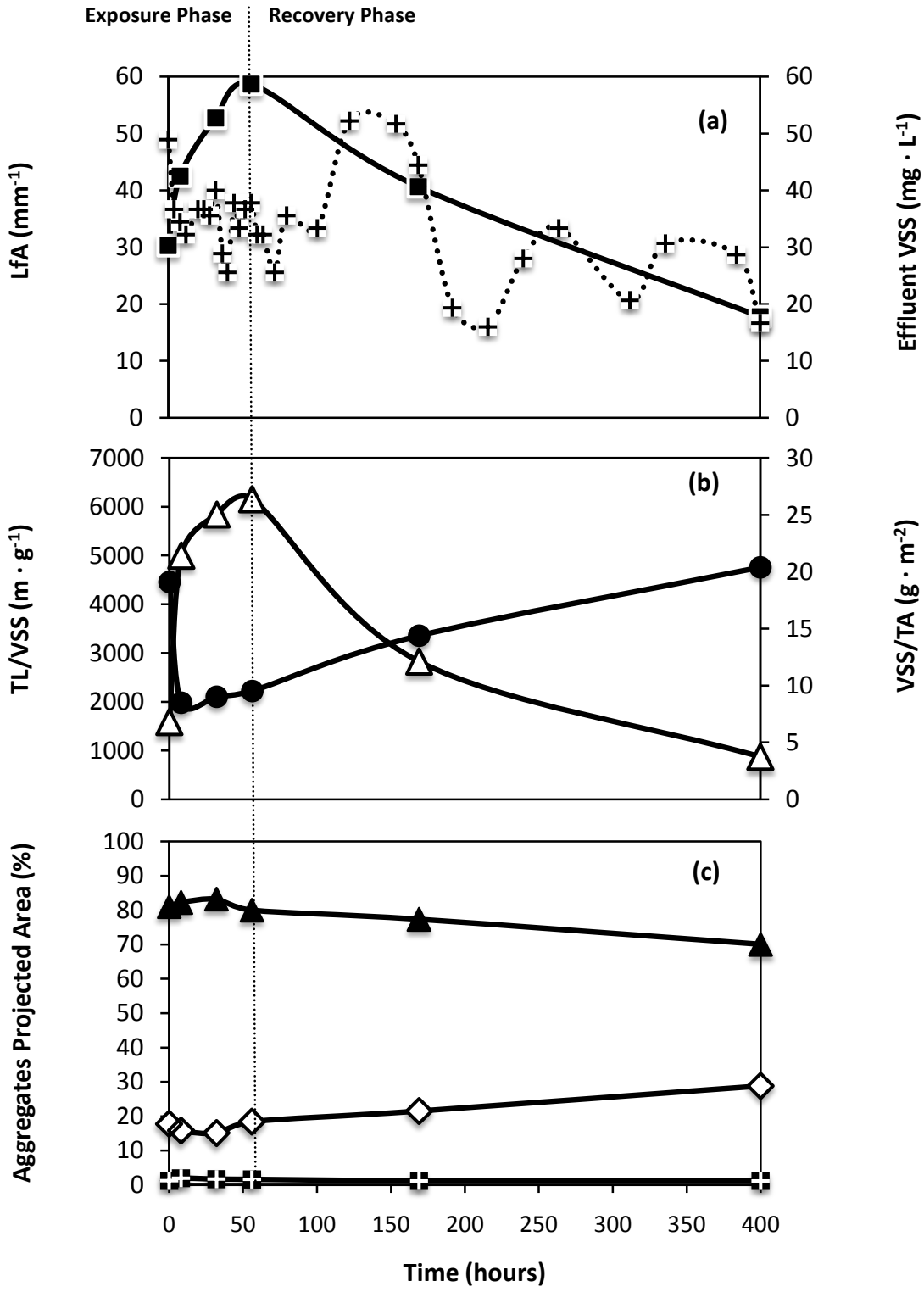


Figure 5.3 – Time course of morphological parameters in shock load I: (a) dynamic between total filament length and total aggregates projected area (LfA, ▬■▬) and effluent volatile suspended solids (Effluent VSS, ▪ ▪╋▪ ▪), (b) total filament length per volatile suspended solids (TL/VSS, ▬△▬) and volatile suspended solids per total aggregates projected area (VSS/TA, ▪ ●▪), and, (c) percentage of projected area for different size classes of aggregates equivalent diameter. ▬▲▬ $D_{eq} \geq$ 1mm; ▬◇▬ 0.1mm ≤ D_{eq} < 1mm; ▬✚▬ 0.01mm ≤ D_{eq} < 0.1mm.

5.3.2 Shock Load with 300 $mg_{COD}·L^{-1}$ of Detergent

A second shock was applied with a higher concentration of detergent (300 $mg_{COD}·L^{-1}$). The exposure time was extended to 222 h. The objective was to study the influence of concentration and exposure time in the anaerobic biodegradation of organic compounds in the presence of detergent/surfactant.

During the operation period, three stages were distinguished, the operation period I (OPI) occurred until the 80th hour, operation period II (OPII) from the 80th hour until the end of exposure phase (222 h), and the operation period III (OPIII) corresponds to the recovery phase, from hour 222 to 505.

5.3.2.1 Operation Period I

The reactor was fed with 1960 $mg_{COD}·L^{-1}$ corresponding to an organic loading rate (OLR) of 6.3 $Kg_{COD}·m^{-3}·d^{-1}$ (Figure 5.4 – OPI). The hydraulic retention time was 7.5 h.

In the first 80 h, the COD removal efficiency was constantly above 75 %, and the effluent COD was roughly constant, around 400 $mg_{COD}·L^{-1}$ (Figure 5.4 – OPI).

It has been reported that the removal of surfactants from the liquid phase is partially due to adsorption at the biomass (Mosche and Meyer, 2002). Mensah and Forster (2003) suggested that the detergent removal from the wastewater is achieved through a combination of adsorption and degradation. Therefore, if we assume that surfactants primarily adsorb to cell membranes (Helenius and Simons, 1975), severe damage of the membrane functions and cell viability can be expected, especially in the transport of nutrients and/or substrate into bacterial cells (Gavala and Ahring, 2002).

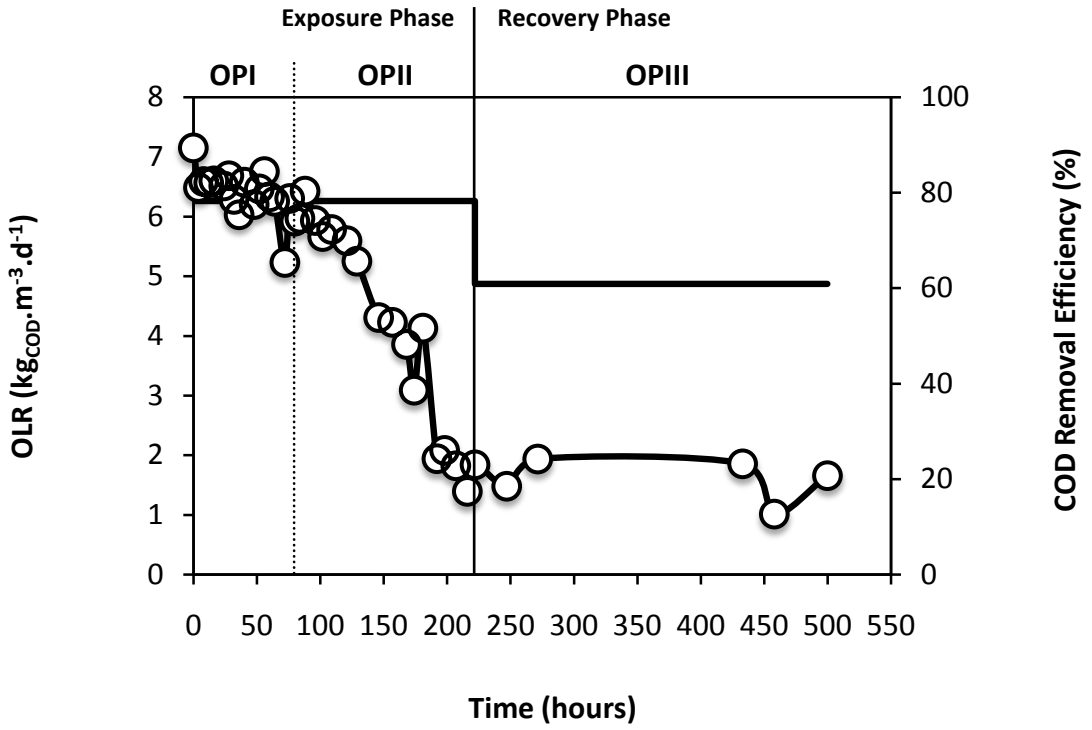


Figure 5.4 – Time course of operational parameters in shock load II: Organic Loading Rate (OLR, ▬), and COD removal Efficiency (⊸).

A detergent concentration of 300 $mg_{COD} \cdot L^{-1}$ immediately started to have an inhibitory effect in the SAA. However, the SHMA was improved in the first 32 h of exposure, suggesting that the hidrogenotrophic bacteria were the less affected by the detergent. During this operation stage the SAA was lost, achieving negligible values in the 80th hour of exposure (Figure 5.5 – OPI). These results indicate that the acetoclastic bacteria were the most affected by the toxic effect of the detergent.

In the beginning of experiment the TL/VSS showed a slight increase caused by filaments release to the bulk. In the next hours of operation TL/VSS decreased possibly because of the free filaments washout (Figure 5.6b – OPI). The granules consistency decreased due to adsorption and self assembly of the surfactants. In fact, the VSS/TA presented a decreasing trend during this period (Figure 5.6b – OPI). Simultaneously, the v_{sed} decreased from 30.6 ± 10.2 to 24.0 ± 13.5 $m \cdot h^{-1}$, proving that the aggregates became fluffier. As expected, in this phase, the LfA parameter

presented the same behaviour as the shock load I, it increased from 18 to 43 mm^{-1} in the first 24 h of operation. After this adapting phase it decreased to 20 mm^{-1}. Simultaneously a small decrease of effluent VSS from 60 to 27 $mg \cdot L^{-1}$ was observed (Figure 5.6a - OPI).

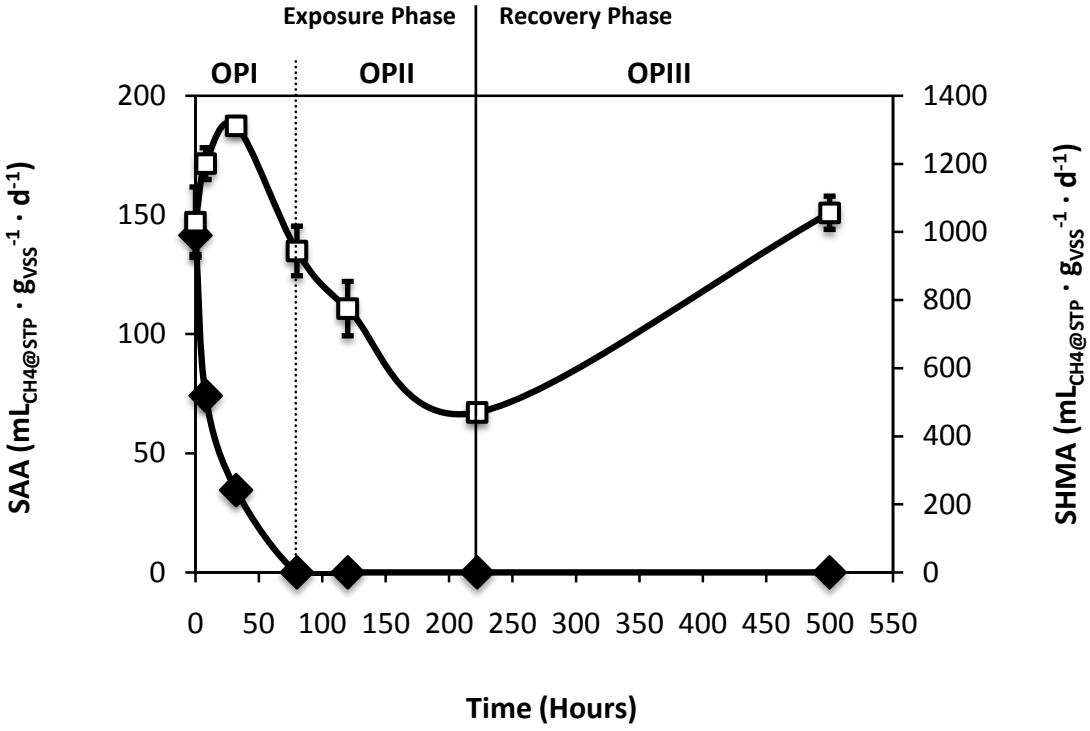


Figure 5.5 – Time course of the specific acetoclastic activity (SAA, ◆), and specific hydrogenotrophic methanogenic activity (SHMA, □), in shock load II.

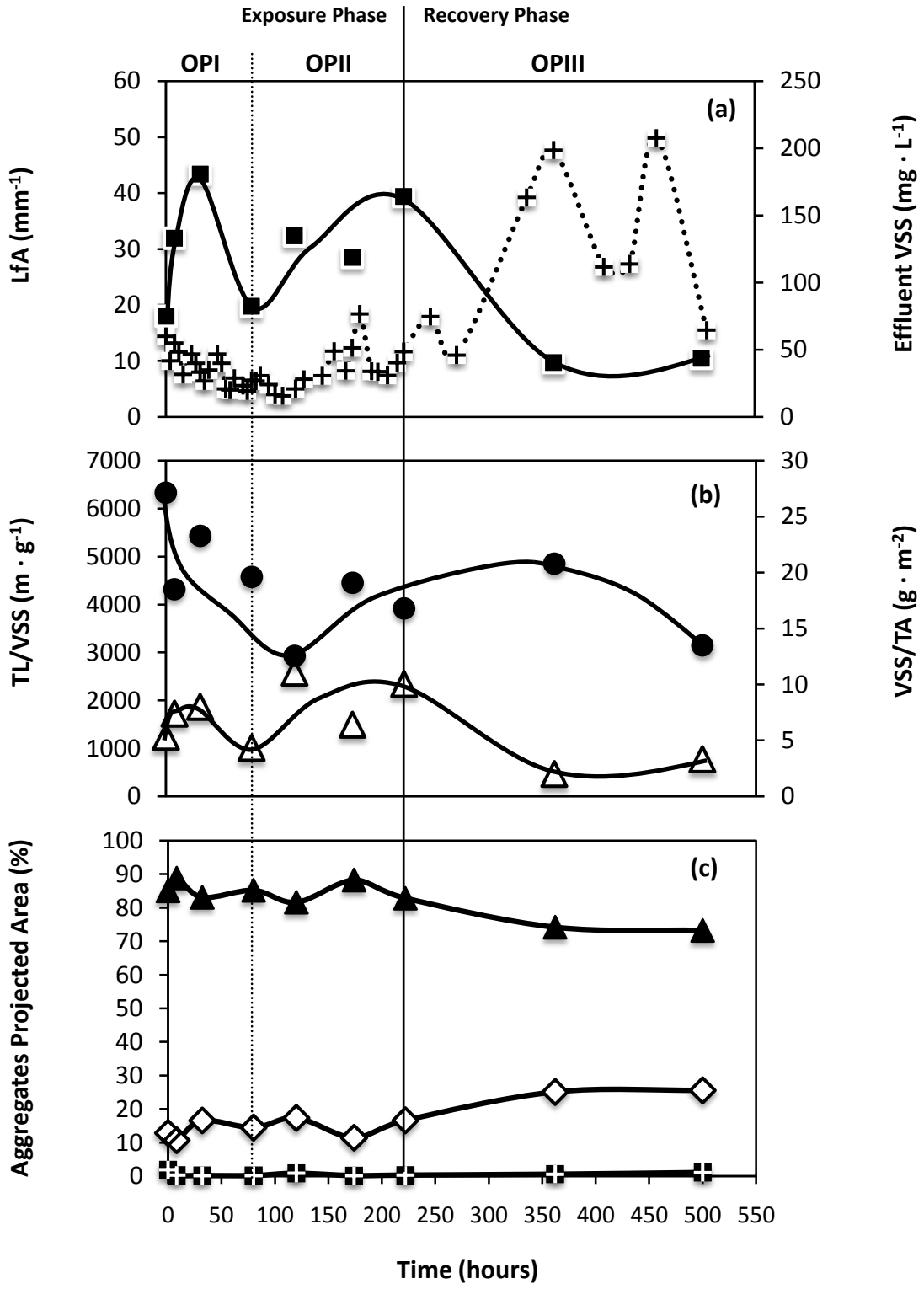


Figure 5.6 – Time course of morphological parameters in shock load II: (a) dynamic between total filament length and total aggregates projected area (LfA, ▬■▬) and effluent volatile suspended solids (Effluent VSS, ▪ ▪✚▪ ▪), (b) total filament length per volatile suspended solids (TL/VSS, ▬△▬) and volatile suspended solids per total aggregates projected area (VSS/TA, ▪ ●▬ ▪), and, (c) percentage of projected area for different size classes of aggregates equivalent diameter. ▬▲▬ $D_{eq} \geq$ 1mm; ▬◇▬ 0.1mm ≤ D_{eq} < 1mm; ▬✚▬ 0.01mm ≤ D_{eq} < 0.1mm.

5.3.2.2 Operation Period II

After the first 80 h of operation, the COD removal efficiency dropped consistently, reaching the value of 17 % at the end of exposure time (Figure 5.4 – OPII). Simultaneously, the methane production was continuously reduced, presenting negligible values at the end of this stage (data not shown), and the effluent COD increased constantly (Figure 5.4 – OPII). It is relevant to note that the loss of performance was acute only after the complete inhibition of the SAA (Figure 5.5 – OPII). Relatively to SHMA a decrease between hours 80 and 222 was observed, inducing the idea that the negative effects of surfactant increased during the exposure time, even to the less sensitive bacteria.

Concerning the image analysis results, it was observed that the effects of detergent shock load on the morphological parameters caused an increase on LfA to 39 mm^{-1} (Figure 5.6a – OPII). This result was a response to the behaviour of filament dynamics inside the reactor. The filaments started to be released from the granules into the bulk, and TL/VSS increased (Figure 5.6b – OPII), inducing the increase of LfA parameter. In this phase, the protruding filaments were in higher length and the increase of effluent VSS was minimal, since only the free filaments and fluffier granules were washed out. Consequently, it was observed that the VSS/TA parameter presented an increasing trend (Figure 5.6b – OPII) because only the denser granules remained inside the reactor.

When the filaments were released the percentages of the smaller aggregates (D_{eq} < 0.1 mm) increased, and, after an occurrence of washout (increase of VSS), these percentages returned close to the initial values (Figure 5.6c - OPII).

5.3.2.2 Operation Period III

In this phase the COD_{in} was reduced to 1530 $mg_{COD}·L^{-1}$, corresponding to an organic loading rate of 5 $Kg_{COD}·m^{-3}·d^{-1}$. During the recovery phase, no improvement in the reactor performance was observed. The COD removal efficiency was persistently lower than 25 % (Figure 5.4 – OPIII). These results suggest that the inhibitory effects caused by 300 $mg_{COD}·L^{-1}$ of detergent, were irreversible during 283h after the end of exposure time.

Relatively to the SAA (Figure 5.5 – OPIII), no recovery was detected during 12 days after exposure. Gavala and Ahring (2002) showed that methanogenesis from hydrogen was affected by the surfactants in much lower range. In this experiment it was observed that, although the SHMA was reduced during the exposure time, in the recovery phase it was completely recovered. This result confirms that hydrogenotrophic bacteria are less affected by the surfactant.

In this phase the effluent VSS increased to values near 200 $mg·L^{-1}$ (Figure 5.6a – OPIII). This fact may be explained by the washout of the small aggregates. Other possible reason of effluent VSS increase was the washout of free filaments, and consequent decrease of TL/VSS (Figure 5.6b – OPIII). These facts induced the reduction of LfA parameter to the lowest value (10 mm^{-1}) (Figure 5.6a - OPIII).

In detergent shock I discussion it was hypothesised that LfA could be an early-warning indicator of biomass washout. The results of this shock load (LfA raise between 80 and 222h and effluent VSS increase, only, after 272h), emphasize this hypothesis.

5.4 CONCLUSIONS

Phenomena of granular erosion, growth and filaments release were identified and quantified, for the first time, during the operational response of an EGSB reactor to detergent shock loads.

The COD removal efficiency was unaffected with 150 $mg_{COD}·L^{-1}$ (56 hours exposure time) of detergent. The SMA was stimulated in the first 8 h of exposure with 150 $mg_{COD}·L^{-1}$ of detergent. After this increase, an inhibitory effect was detected. Nevertheless, the SMA was recovered 5 days after the end of exposure time. However 80 h after exposure to 300 $mg_{COD}·L^{-1}$ of detergent, the COD removal efficiency decreased from 75 % to 17 %. In this experiment, significant changes in the specific methanogenic activity and granular sludge morphology were detected immediately after exposure to surfactant (300$mg_{COD}·L^{-1}$), whereas operational performance only deteriorated after 80 hours of exposure time. It is relevant to note that in these conditions, the loss of performance was acute only after the complete inhibition of the specific acetoclastic activity. The acetoclastic bacteria were the most sensitive to the inhibitory effect of surfactant and the hydrogenotrophic bacteria the least sensitive. The inhibitory effects were dependent on surfactant concentration and exposure time.

Image analysis techniques allowed quantifying the structural stability of anaerobic granular sludge. Both detergent shocks induced an immediate increase of free filaments in the bulk medium, from 1600 to 6150 $m·g^{-1}_{VSS}$ and from 1200 to 1850 $m·g^{-1}_{VSS}$, in the 150 $mg_{COD}·L^{-1}$ and in the 300 $mg_{COD}·L^{-1}$ detergent shock, respectively. No significant size reduction was observed in the larger aggregates in both shocks.

The filaments length/total aggregate area (LfA) parameter confirmed to be sensitive to detect changes in the sludge morphology. LfA increased 3 and 5 days before the detection of a severe washout event, in shock load I and II, respectively, suggesting

that in the present experiment it could have been an early-warning indicator of biomass washout.

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1 2 3 4 5 6 7 8

Solvent Shock Load

ABSTRACT

The use of quantitative image analysis techniques, together with physiological information might be used to monitor and detect operational problems in advance to reactor performance failure. Industrial organic solvents such as *white spirit* are potentially harmful to granular sludge. In preliminary batch assays, 33 $mg \cdot L^{-1}$ of solvent caused 50% relative biomass activity loss. In an Expanded Granular Sludge Blanket reactor fed with 40 $mg \cdot L^{-1}$ of solvent, during 222 h, the reactor performance seemed to be unaffected, presenting COD removal efficiency consistently above 95 %. However, in the last days of exposure, the biogas production and the methane content were inhibited. Afterwards, already during recovery phase, the COD Removal Efficiency decreased to 33 %, possibly because the reactor was underloaded and the biomass became saturated in solvent only in this stage. In the first hours of exposure the specific acetoclastic and the specific hydrogenotrophic methanogenic activities decreased 29 and 21 %, respectively. The % of aggregates projected area with equivalent diameter (D_{eq}) > 1 mm decreased from 81 to 53 %. The mean D_{eq} of the aggregates larger than 0.2 mm decreased, as well as the settling velocity, showing that the granules experienced fragmentation phenomenon caused by the solvent shock load. The ratio between total filaments length and total aggregates projected area (LfA) increased 2 days before effluent volatile suspended solids, suggesting that LfA could be an early-warning indicator of washout events.

6.1 INTRODUCTION

Hydrocarbons are the product of incomplete combustion of organic substances such as fossil fuel, wood and mineral oil. Hydrocarbons can be classified into three classes. Aromatic hydrocarbons, also known as arenes, have at least one aromatic ring. Saturated hydrocarbons, also known as alkanes or aliphatic hydrocarbons don't have any double, triple or aromatic bonds, their formula are C_nH_{2n+2}. Finally, unsaturated hydrocarbons have one or more double or triple bonds between carbon atoms. Those with one double bond are called alkenes, with the formula C_nH_{2n} (assuming non-cyclic structures). Those with only one triple bond are called alkynes.

Organic solvents comprise a group of various liquid hydrocarbons obtained from the intermediate products of the crude distillation. They are used in the chemical industry as solvent for extraction, cleaning and degreasing, and, in aerosols, paints, wood preservatives, lacquers, varnishes, and asphalt products. The destiny of petroleum type pollutants in the environment has been investigated in many studies (Antic *et al.*, 2006). Also, the biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and marine ecosystems (Margesin and Schinner, 2001). Organic solvents are flammable, malodorous and potentially toxic to aquatic organisms and thus require complete elimination by wastewater treatment systems (Henry et al., 1996). Due to the hydrophobic nature of hydrocarbons they are mostly bound to the sludge and escape aerobic treatment in a wastewater treatment plant making them present in the anaerobic post treatment (Christensen et al., 2004; El-Hadj et al., 2006). It is nowadays undeniable that various toxic organic compounds, such as surfactants

and hydrocarbons, like the organic solvent *White Spirit* (McGovern *et al.*, 2002) are found in sewage and industrial wastewaters. A review of the toxicology of mineral spirits, expanding the existing database on the toxicology of this group of hydrocarbon solvents can be found in (Amoruso et al., 2008).

Wastewater containing solvents poses a risk to biological treatment systems (Inanc et al., 2002), with emphasis in anaerobic digesters because of their high biomass adsorption capability. Less than 20 years ago it was a generally accepted idea that hydrocarbons, except for comparably reactive ones such as acetylene, could not be degraded in the absence of molecular oxygen (Schink, 2002). Meanwhile, studies have reported the degradation of hydrocarbons in anaerobic systems. Spormann and Widdel (2000) present a review focused on the anaerobic degradation of aromatic and saturated hydrocarbons. An anaerobic completely stirred tank reactor fed with pharmaceutical wastewater, experienced a dramatic deterioration in performance in terms of COD removal efficiency (almost none) and acetoclastic methanogenic activity (less than 10 $mL_{CH4} \cdot g_{TVS}^{-1} \cdot d^{-1}$) and a significant increase in VFA concentration (Akarsubasi et al., 2005). Enright et al. (2005) used expanded granular sludge bed reactors for the treatment of solvent contaminated wastewater at 15 ºC, and achieved COD removal efficiencies of 60–70%. However, despite current knowledge about the anaerobic digestion process, little is known about the effects caused by solvents in anaerobic granular sludge.

Combination of accurate morphological parameters, given by quantitative image analysis at micro and macro structure levels, with physiological activity of anaerobic granular sludge and reactor performance, may provide pertinent information about the stability of high rate anaerobic reactors in the presence of potential disturbances. The usefulness of this methodology in the detection of operational problems was already demonstrated for anaerobic granular sludge in contact with detergent/surfactant (Costa *et al.*, 2007). Like surfactants, solvents can disrupt the efficient functionality of anaerobic digesters when a redundant failure occurs because of its widespread use in industry. This work focuses on the study of effects

caused by the exposure of anaerobic granular sludge to the organic solvent *White Spirit*, which is the most widely used solvent in the paint industry.

6.2 EXPERIMENTAL SECTION

The operating parameters, specific methanogenic activity methodology, and image analysis technique used during these experiments are described in Chapter 3 (Methodology) of this thesis.

6.2.1 Solvent characterization

The solvent tested is a paraffin-derived, transparent liquid, which is a common organic solvent used in extraction, cleaning and degreasing industrial processes. It is a mixture of saturated aliphatic and alicyclic C7 to C12 hydrocarbons with a maximum content of 25% of C7 to C12 alkyl aromatic hydrocarbons. The solvent presents a density (at 15°C) of about 0.785 $g \cdot m^{-3}$.

6.2.2 Toxicity Test

The effect of an inhibitor in the methanogenic activity of a specific trophic group may be determined following the increase of biogas, in sealed vials.

After biomass acclimation at 37ºC and 150 rpm, toxicity batch assays were performed by adding acetate (30 mM) and increasing solvent concentrations (1.6, 7.9, 39.3, and 78.5 $mg \cdot L^{-1}$) to the sludge, in batch vials. Working volume was 12.5 ml and total volume was 25 ml. Fifty percent inhibition concentration (IC_{50}) was

defined as the solvent concentration that caused 50% relative acetoclastic activity loss. All batch experiments were performed in triplicate assays.

6.2.3 Inoculum and substrate

The EGSB reactor was inoculated with 400 mL of granular sludge. The biomass was characterised in terms of specific methanogenic activity (SMA) with acetate and H_2/CO_2 as substrates, morphological parameters assessed by quantitative image analysis, volatile suspended solids (VSS), and settling velocity (Table 6.1).

The reactors were fed with 1.5 $g_{COD}·L^{-1}$ of ethanol. Sodium bicarbonate was added as the alkalinity source (2 $g·L^{-1}$). Micro and macronutrients were added according to:

Macronutrients – $MgSO_4·7H_2O$: 30 $g·L^{-1}$; KH_2PO_4: 28.3 $g·L^{-1}$; NH_4Cl: 170 $g·L^{-1}$. 0.6 mL of this solution was added per gram of COD fed.

Micronutrients – $FeCl_2·6H_2O$: 2$g·L^{-1}$; H_3BO_3: 0.05 $g·L^{-1}$; $ZnCl_2$: 0.05 $g·L^{-1}$; $CuCl_2·2H_2O$: 0.038 $g·L^{-1}$; $MnCl_2·4H_2O$: 0.5 $g·L^{-1}$; $(NH_4)_6Mo_7O_{24}·4H_2O$: 0.05 $g·L^{-1}$; $AlCl_3·6H_2O$: 0.09 $g·L^{-1}$; $CoCl_2·6H_2O$: 2 $g·L^{-1}$; $NiCl_2·6H_2O$: 0.092 $g·L^{-1}$; $Na_2SeO_3·5H_2O$: 0.164 $g·L^{-1}$; EDTA: 1 $g·L^{-1}$; Resazurin: 0.2 $g·L^{-1}$; HCl 37%: 1 $mL·L^{-1}$. The composition of this solution was based on the work of Zehnder *et al.* (1980) and was supplemented to the influent feed by addition of 1 ml per litre.

When the reactor was operating in steady-state, with stable values, either in terms of Chemical Oxygen Demand (COD) removal efficiency, specific methanogenic activity (SMA) or morphological parameters, the solvent (40 $mg·L^{-1}$) was mixed with the feeding, with constant agitation, during 222 hours. The recovery phase was followed through 7 days (Table 6.1).

Table 6.1 – Inocula characterization and shock load conditions.

SHOCK LOAD	
Inoculum Characterization:	
Specific Acetoclastic Activity ($mL_{CH4@STP} \cdot g_{VSS}^{-1} \cdot d^{-1}$)	212 ± 27
Specific Hydrogenotrophic Methanogenic Activity ($mL_{CH4@STP} \cdot g_{VSS}^{-1} \cdot d^{-1}$)	910 ± 85
Morphology	
LfA (mm^{-1})	24
TL/VSS ($m \cdot g^{-1}$)	1800
VSS/TA ($g \cdot m^{-2}$)	13
VSS ($g \cdot L^{-1}$)	26.5
Settling velocity ($m \cdot h^{-1}$)	26.0 ± 14.0
Shock Load Conditions:	
Ethanol ($mg_{COD} \cdot L^{-1}$)	1500
Solvent ($mg \cdot L^{-1}$)	40
Exposure Time (h)	222
Recovery Phase (days)	7

6.3 RESULTS AND DISCUSSION

6.3.1 Toxicity Test

Previously to exposure in a lab-scale reactor, a series of batch assays was performed to determine the concentration of solvent that caused 50% relative activity loss (IC_{50}). The Specific Acetoclastic Activity (SAA) decreased proportionally to the solvent concentration increase. The IC_{50} was 33 $mg \cdot L^{-1}$.

6.3.2 Reactor Performance

The EGSB reactor fed with 4.8 $kg_{COD}·m^{-3}·d^{-1}$ and hydraulic retention time of 8 hours, achieved COD removal efficiency higher than 95%. When the reactor showed stable performance was exposed to 40 $mg·L^{-1}$ of solvent. During the exposure phase the OLR increased to 6 $kg_{COD}·m^{-3}·d^{-1}$ (Figure 6.1). The reactor performance seemed to be unaffected by the shock load, since the COD removal efficiency remained, constantly, above 95 %. However, during the last two days of exposure, the biogas production and methane content decreased to residual values (data not shown).

In the first 3 days of recovery phase, the COD removal efficiency decreased, reaching a minimum of 33 % (Figure 6.1). Simultaneously, the effluent volatile fatty acids increased, in particular acetate with concentrations of 500 $mg_{COD}·L^{-1}$ (data not shown). Several possible explanations can justify this later decrease of the COD removal efficiency: a) Urra et al. (2008) studied the influence of the biomass concentration in the level of inhibition and the anaerobic degradation kinetics resulting from the incorporation of toxic compounds (long chain fatty acids, polycyclic aromatic hydrocarbons, linear alkylbenzene sulphonates and organic acids) in an anaerobic system. They observed a link between the tolerance to toxic compounds and the biomass concentration. In addition, it was observed that the degradation kinetics is affected, whether diminishing the methane production (polycyclic aromatic hydrocarbons, linear alkylbenzene sulphonates, organics acids) or increasing the initial latency time (long chain fatty acids). In the present study, once the OLR was too small for an EGSB reactor, i.e. the reactor was underloaded, the negative effects on the reactor performance were postponed, because there was enough biomass to degrade the ethanol. b) In the batch assays the solvent was in direct and constant contact with the biomass, however, in continuous operation the contact was not so intensive. During the reactor operation, the solvent accumulation in a top layer of the reactor was observed. The solvent short-circuited through the reactor, possibly because of its small density, causing a temporary shift in the concentration in direct and constant contact with the biomass. This

concentration is smaller than the effectively fed. Afterwards, this top layer was recirculated causing a punctual increase of the solvent concentration entering in the reactor. This higher concentration might be responsible for the reactor performance decrease. c) As stated before hydrocarbons have the tendency to bind onto the biomass (Christensen *et al.*, 2004). The granular sludge was 222 hours exposed to solvents. These caused its saturation with consequent granules fragmentation and biomass washout. Since the biogas production and methane content decreased, while acetate concentration increased, and the ethanol concentration remained undetected in the effluent, is clear that the methanogenesis was inhibited. It is possible to speculate that the Methanosaeta genera, which are a very slow growing archaea could have been selectively washed out, as already observed during organic loading disturbances (Costa et al., 2009).

Ninety hours after shock load was stopped, the reactor recovered previous COD removal efficiencies of ≥ 95% (Figure 6.1). The biogas flow and the methane content, also increased to values close the initial ones (data not shown). Once the shock load was already stopped when the negative effects became visible, the reactor was capable of recover. According to [20], if the testing period is not enough, there is the risk of not fully developing the degradation kinetics causing us to assume erroneous inhibition values. In this study, the exposure time was not enough to cause an irreversible inhibition in the biomass. It is possible to conclude that the EGSB reactor is capable of resist to a punctual contamination with a small concentration of solvent. However, if the exposure time or the solvent concentration is high is probable that the reactor performance will decrease drastically.

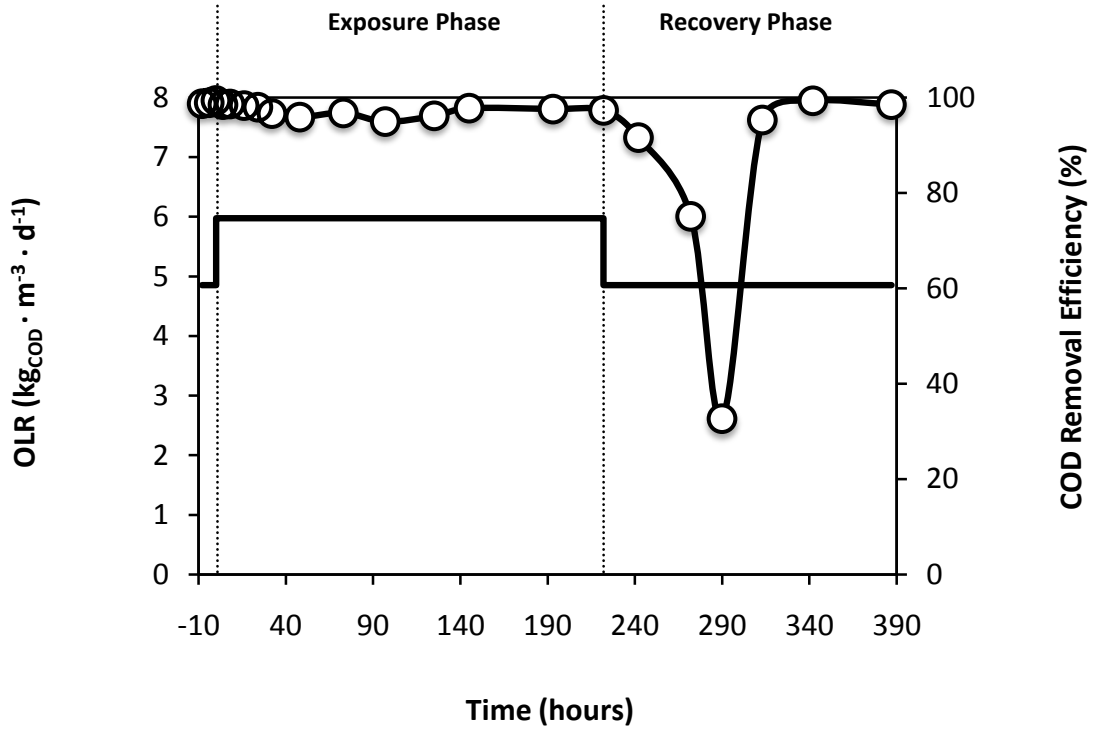

Figure 6.1 – Time course of organic loading rate (OLR, ▬), and COD removal efficiency (⊸).

6.3.2 Specific Methanogenic Activity

The effects on the specific methanogenic activity (SMA) were not significant as could be expected after the batch assays results. Nonetheless, during the first 24 hours of exposure, the solvent shock load caused a decrease of 21% in the SHMA (Figure 6.2). As for the SAA a continuous decrease was observed during the first 73 hours of shock load, with 29% of inhibition (Figure 6.2). The acetoclastic bacteria, which were protected inside the granules, became more vulnerable to the solvent effects, because of granule disintegration (Figure 6.6c). Subsequently the SHMA and the SAA stabilised around 760 and 150 $mL_{CH4@STP} \cdot g_{VSS}^{-1} \cdot d^{-1}$, respectively, until the end of the exposure phase.

During recovery phase, a sudden increase in the SMA was observed (Figure 6.2). This observation was unexpected because during the same period the reactor performance decreased. It is possible that the granules fragmentation facilitate the transport of the substrate to the bacteria inside the granules causing a momentary increase of the SMA. The SMA increase with the decrease of mass transfer limitations between the bulk and the cores of the newly formed small particles after fragmentation was already stated by Abreu et al. (2007).

6.3.3 Image Analysis

The main effect caused by the shock load was the granules fragmentation. Figure 6.3 shows several granules breaking, with resulting decrease in size and filaments release.

The mean macroflocs equivalent diameter (D_{eq}) decreased consistently from 0.9 to 0.6 mm (Figure 6.4). Figure 6.5 shows original and respective binary images of granules, from the inoculum, at the middle of operation (t = 125 h), and in the end of exposure, supporting the previous statement. Thus, the decrease in granules size is clearly observed. The constant binding of hydrocarbons to cell membranes caused severe damages, and can explain the aggregates fragmentation/erosion with a consequent decrease of D_{eq} and filaments release. Consequently, the % of projected area of aggregates with equivalent diameter (D_{eq}) higher than 1 mm decreased from 81 to 53 % during exposure phase, and, accordingly, the % of projected area of small aggregates ($0.1 \leq D_{eq}$ (mm) < 1) increased from 18 to 46 % (Figure 6.6c).

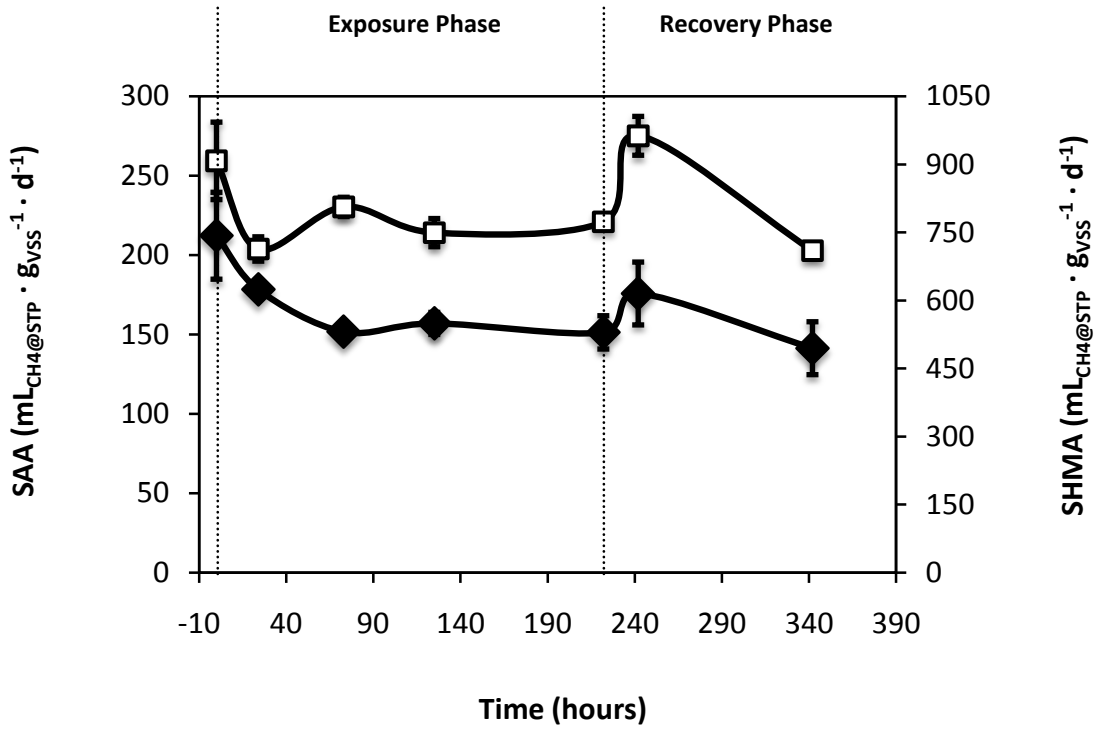

Figure 6.2 – Time course of specific acetoclastic activity (SAA, ◆), and specific hydrogenotrophic methanogenic activity (SHMA, □).

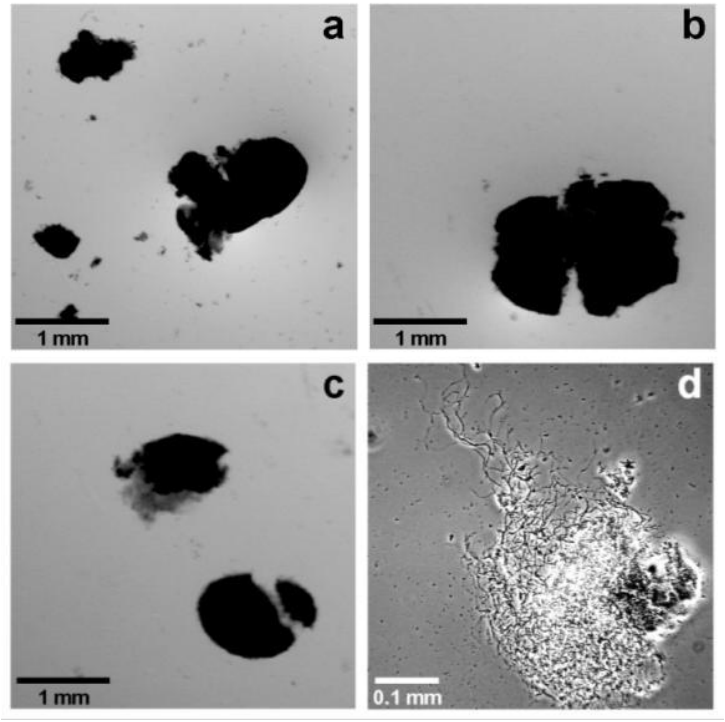

Figure 6.3 – Images of granules fragmentation and filaments release during exposure to solvent.

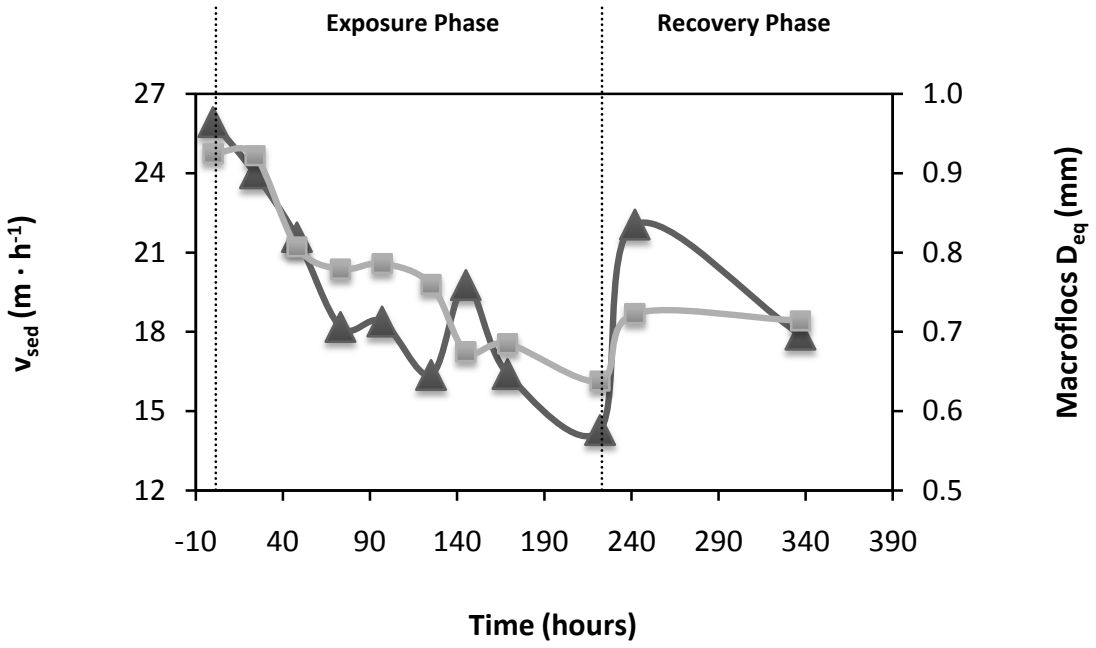

Figure 6.4 – Time course of averages of settling velocity (v_{sed},), and macroflocs equivalent diameter ( ).

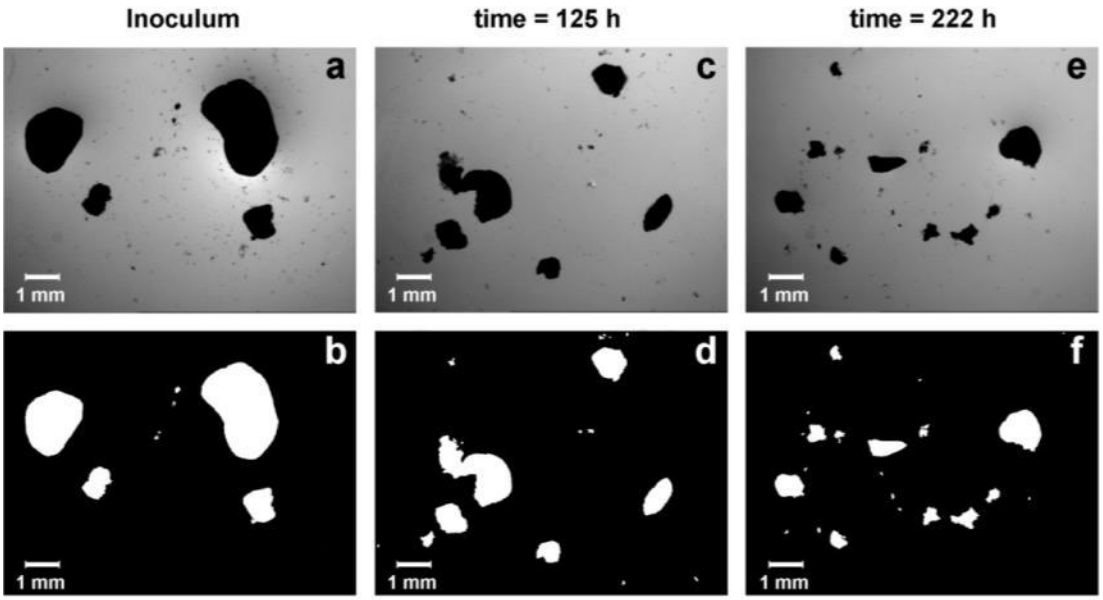

Figure 6.5 – Original (a, c, e) and binary (b, d, f) images of granules from the inoculum, after 125 of exposure, and in the end of shock load (222 h).

During the first hours of exposure, the TL/VSS decreased, possibly because of the free filaments washout. Afterwards, as a consequence of granules fragmentation/erosion the filaments release occurred, as observed in the increase of TL/VSS from 1300 to 2000 $m \cdot g^{-1}$, 40 hours after the exposure (Figure 6.6b). However, during this phase the biggest part of filaments are still connected to granules (protruding filaments) as can be observed in Figure 6.3d. During this stage the higher increase in LfA parameter occurs (Figure 6.6a). Later, when the filaments are completely free in the bulk, the washout occurs, corresponding to the highest peak of effluent volatile suspended solids (VSS) (Figure 6.6a). Consequently the LfA and TL/VSS decreased to 14 mm^{-1} and 1100 $m \cdot g^{-1}$, respectively.

As already reported in Amaral *et al.* (2004) and Costa *et al.* (2007), the LfA parameter was, also in this case, able to be an early-warning indicator of washout events during the solvent shock load. Effectively, during this experiment the LfA increased 35 % between hours 24 and 73 (Figure 6.6a), reaching the value of 29 mm^{-1}. In the same figure, is showed that the effluent volatile suspended solids (VSS), only achieved its maximum value at 125 h, *i.e.* 52 hours after LfA.

At the end of exposure phase, 145 hours after the effluent VSS decrease, it was observed a new peak of 82 $mg \cdot L^{-1}$, suggesting a washout of small aggregates. This hypothesis was enhanced by the % of aggregates projected area with $D_{eq} \geq 1$ mm increase (Figure 6.6c) during the first hours of recovery phase.

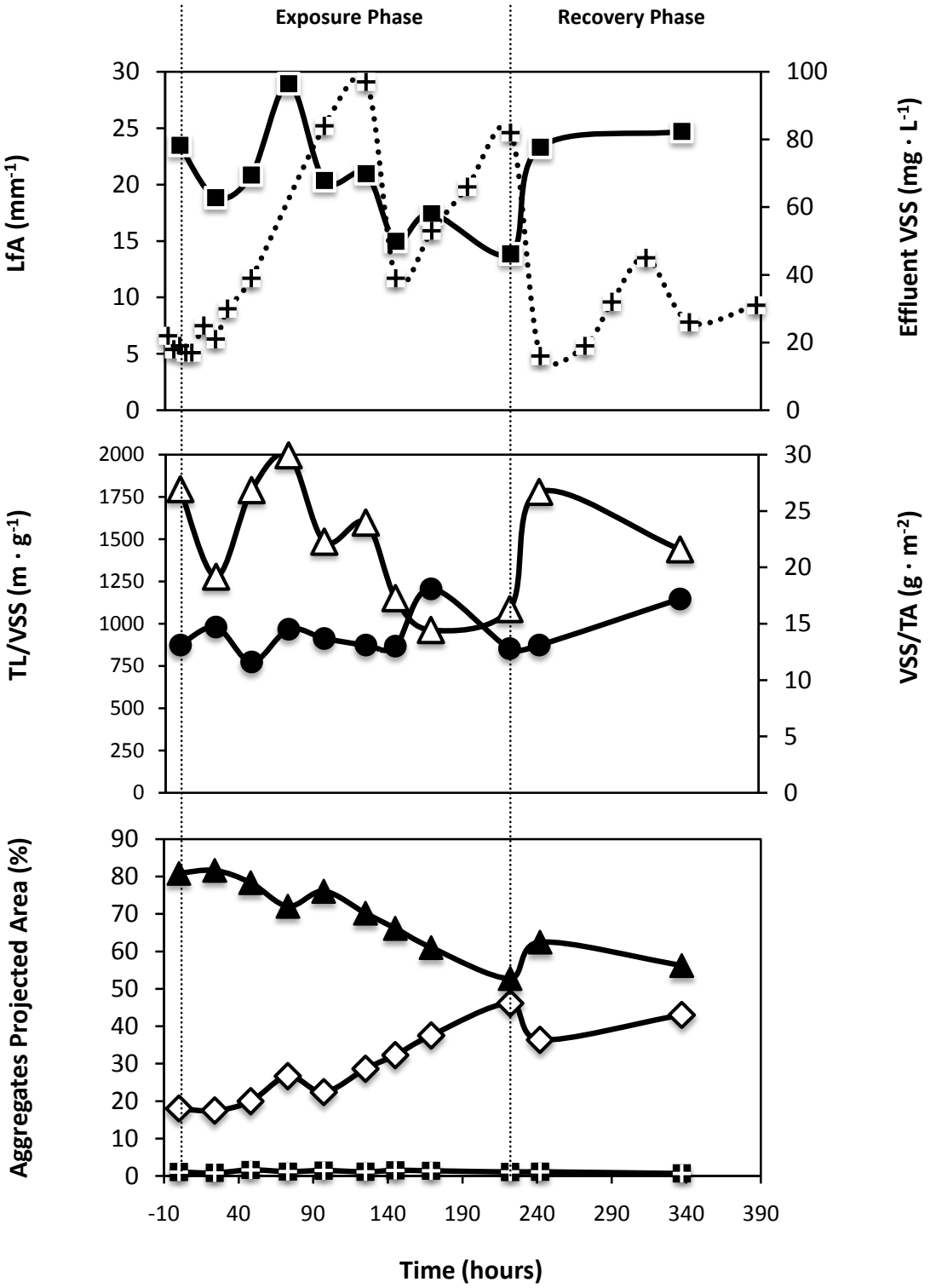

Figure 6.6 – Time course of morphological parameters: (a) dynamics between total filament length and total aggregates projected area (LfA, ▬■▬), and effluent volatile suspended solids (effluent VSS, ▪▪╋▪▪); (b) total filament length per volatile suspended solids (TL/VSS, ▬△▬), and volatile suspended solids per total aggregates projected area (VSS/TA, ▪●▪); (c) percentage of projected area for different size ranges of aggregates equivalent diameter (▬▲▬ $D_{eq} \geq 1$ mm, ▬◇▬ $0.1 \leq D_{eq}$ (mm) < 1, ▬╋▬ $0.01 \leq D_{eq}$ (mm) < 0.1).

A decrease in the mean settling velocity (v_{sed}) from 26 to 14 $m \cdot h^{-1}$ was observed during exposure phase (Figure 6.4). However, the apparent granules density (VSS/TA) was almost constant, around 13 $g.m^{-2}$ (Figure 6.6b). Therefore, the granules fragmentation/erosion may have caused the v_{sed} decrease, as observed by the similar trends of the averages of settling velocity and macroflocs D_{eq} in Figure 6.4. Figure 6.3 and Figure 6.5 show that the granules became more irregular and less robust potentially contributing for the settling velocity decrease.

6.4 CONCLUSIONS

In batch assays it was determined that 33 $mg \cdot L^{-1}$ of solvent caused 50% loss of specific acetoclastic activity. Nonetheless, the overall reactor performance was unaffected by a *White Spirit* shock load of 9 days. The COD removal efficiency was consistently higher than 95% during the exposure phase, probably because the reactor was underloaded and the negative effects were postponed. However, in the last days of exposure a decrease in biogas production and methane content was already observed. During the recovery phase the efficiency decreased to 33 %, possibly due to the accumulation and saturation of solvent onto the biomass or the temporary increase in solvent concentration. However, the reactor rapidly recovered its high efficiency indicating temporary inhibition. A decrease of 21% was observed in the Specific Hydrogenotrophic Methanogenic Activity whereas the Specific Acetoclastic Activity decreased 29% a few hours after initial exposure.

Phenomenon of granular erosion and/or fragmentation and filaments release were identified and quantified, during the exposure of anaerobic granular sludge to an organic solvent. The % of aggregates projected area with $D_{eq} \geq 1$ mm decreased from 81 to 53 %. The average settling velocity (v_{sed}) decreased from 26 to 14 $m \cdot h^{-1}$, although the VSS/TA was almost constant through the exposure phase,

symptomatic that the granules density remained approximately unchanged. The decrease in settling velocity was mainly caused by the decrease in size as a result of granules erosion/fragmentation. The peak in effluent VSS was observed 52 hours after LfA increased 35 %, suggesting that it could be used as an early-warning indicator of washout events when anaerobic granular sludge is exposed to organic solvents such as *White Spirit*.

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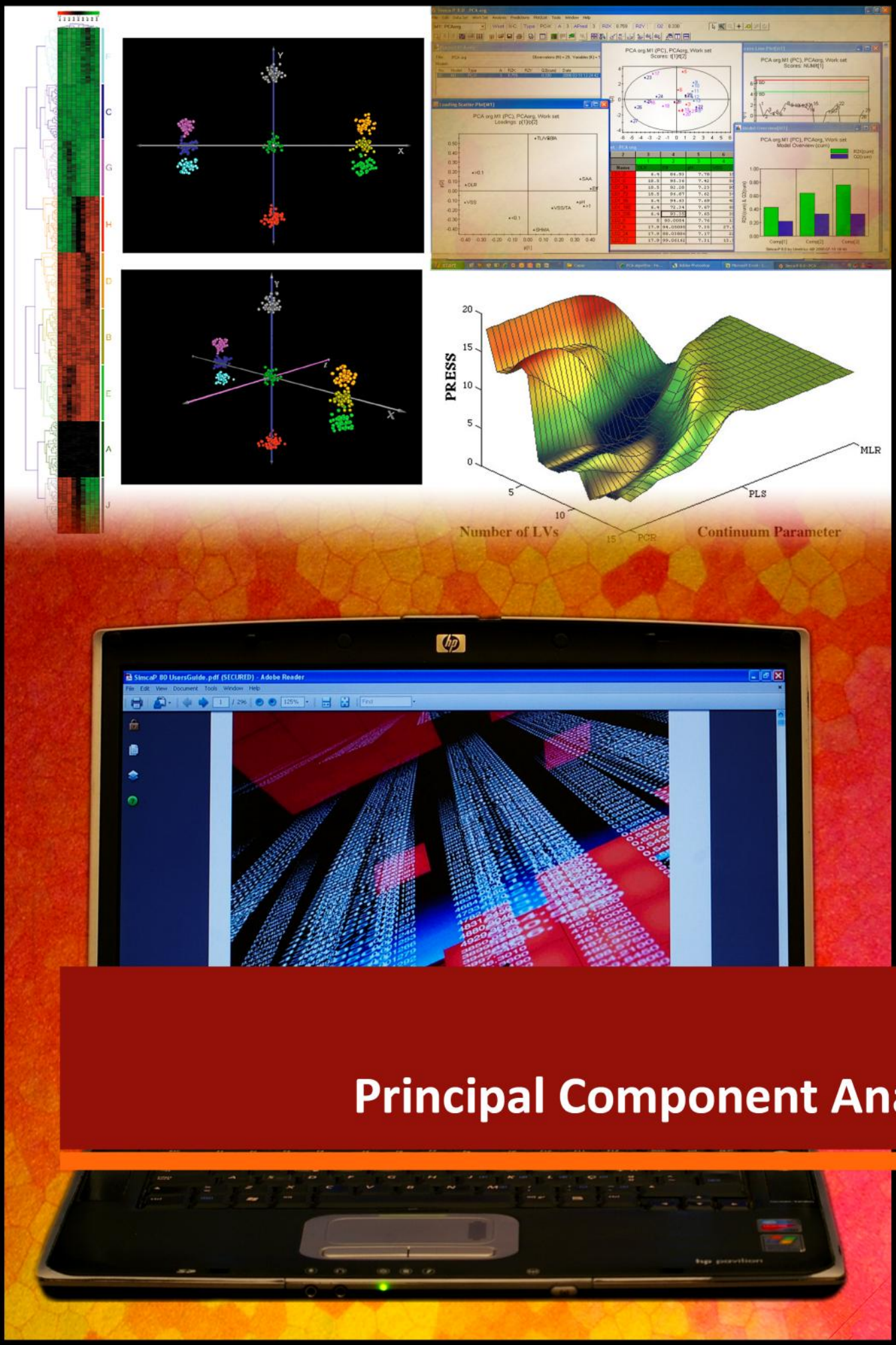

Principal Component Analysis

1 2 3 4 5 6 7 8

ABSTRACT

During the last decade, reports about monitoring and control of anaerobic wastewater treatment processes do not consider the integration of quantitative morphological indicators. The application of Principal Components Analysis (PCA) to datasets obtained during four load disturbances and three toxic shock loads determined a latent variable that encompasses a weighted sum of performance, physiological and morphological information. This new variable was highly sensitive to reactor efficiency deterioration, enclosing remarkable variation in the first hours of disturbances. The high loadings raised by image analysis parameters revealed that morphological changes of solid phase (biomass) should be considered to monitor load disturbances and toxic events in high rate anaerobic (granular) sludge bed digesters. The extraction of two principal components (PC) allowed the visualization of the main effects caused by the disturbances, coupling score (t[1] vs. t[2]) and loading (p[1] vs. p[2]) maps. The plane drawn by PC1 and PC2 grouped the samples according to its operating conditions. A key advantage of PCA is the possibility to visualize all data in a reduced dimensional space, allowing the finding of hidden correlations among variables. Emphasis should be given to the fact that granule aggregation state is highly correlated with the filaments dynamics inside the reactor.

7.1 INTRODUCTION

Stable operation of high-rate anaerobic reactors is an essential but difficult task because of the complicated nature of the anaerobic process itself. Monitoring and control are therefore extremely important to improve process robustness by detecting disturbances leading to abnormal process operation. In this context, identification of process variables potentially useful as early alert detectors of instability has major relevance. Small changes in concentrations or flows can have a large effect on the kinetics of biological reactions, leading to process performance deterioration (Lee et al. 2006). The earlier a potential fault is detected, the less severe its influence will be and the corresponding corrective action will be more constrained.

An important factor for the efficient operation of anaerobic processes, extensively studied in the last decade, is the recognition of parameters that could be used for monitoring/control the process. For automatic monitoring and control, parameters in the solid phase are not often used since they usually need manual operations, and are usually qualitative and inaccurate. Therefore, so far, parameters used for control have been limited to indicators of the liquid and the gaseous phases, such as pH, Volatile Fatty Acids (VFA), alkalinity, COD concentrations, carbon dioxide, methane and hydrogen contents in the biogas as well as biogas production (van Lier et al., 2001). Voolapalli and Stuckey (1998) reported that a combination of physical and biological methods is effective in minimizing process imbalances in anaerobic digesters during intense organic shock loads. In this framework, quantitative image

analysis techniques emerge as promising tool to overcome these difficulties, providing quantitative parameters of the solid phase evolution.

Because the experimental approach of integrating reactor performance, physiological and morphological data may produce huge amounts of correlated and redundant data, a statistical instrument should be applied in order to extract the essential information for process monitoring and fault detection applications. Often, important information lies not in any individual variable but in how the variables change with respect to one another, *i.e.* how they co-vary (Wise and Gallagher, 1996). Data reduction and interpretation can be approached through the application of multivariate statistical methods, such as Principal Components Analysis (PCA). This method allows identifying patterns in data, and expressing them in order to highlight their similarities and differences. PCA is a projection method for analyze data and reduce it from an n-dimensional space to few latent/hidden variables (Lee et al., 2006), while keeping information on its variability.

PCA has been successfully applied to the monitoring of industrial processes (MacGregor and Koutodi, 1995; Li et al., 2000) and wastewater treatment processes (Rosen, 2001; Lee and Vanrolleghem, 2004; Lee et al., 2004). Multivariate statistical analysis has been used together with image analysis techniques to pattern recognition, such as discriminant analysis, neural networks, and decision trees (Ginoris et al., 2007). The relationships between morphological parameters and biomass properties in aerobic wastewater treatment processes were also assessed by PLS (Amaral et al., 2005) and PCA (Jenné et al., 2006).

Since patterns in data can be hard to find in data of high dimension, where graphical representation is not available, the possibility of grouping the variability in few variables is an important step to visualize and consequently analyze the information. Chemometrics based techniques are vehicles that can lead chemists to move more efficiently on the path from measurements to information to knowledge (Frank and Kowalski, 1982).

In chapters 4 to 6 of this thesis are described the effects caused by four organic load disturbances and three toxic shock loads applied to lab-scale Expanded Granular Sludge Bed (EGSB) reactors. The corresponding effects were monitored by quantitative image analysis, specific methanogenic activity tests and reactor performance. In the present study, the multivariate statistical tool Principal Components Analysis was applied to integrate all information in order to achieve a timely monitoring of the process, with early recognition of operational problems and recovery states. In addition, PCA was employed to visualize correlations between morphological, physiological and operational parameters, in order to identify the variables that mostly reflect the load disturbances and toxic shock loads, and respective operational changes/problems recognition.

7.2 EXPERIMENTAL SECTION

In load disturbances LD1 and LD2, the organic loading rate (OLR) was increased from 5 to 18.5 $kg_{COD}·m^{-3}·d^{-1}$, through the increase of ethanol concentration and feed rate, respectively. In LD3 and LD4, the OLR was increased to 50 $kg_{COD}·m^{-3}·d^{-1}$, during 3 and 16 days, respectively. In toxic shock load SL1 and SL2, a commercial detergent was fed to the reactor with a concentration of 1.6 and 3.1 $mg·L^{-1}$, respectively. SL3 was performed by adding 40 $mg·L^{-1}$ of a solvent to the EGSB reactor. Variables summarizing the morphological, physiological and performance data obtained during organic loading disturbances and toxic shock loads were grouped to create the datasets used to perform the PCA (Table 7.1). Seven datasets were created, one for each load disturbance and toxic shock load. Another three datasets were created gathering the results of load disturbances, toxic shock loads, and all available data, respectively.

Table 7.1 – Variables included in dataset, summarizing the changes occurred during load disturbances and toxic shock loads

Variable	Name
Reactor Performance Data:	
OLR	Organic Loading Rate
HRT	Hydraulic Retention Time (LD2)
Cdet	Detergent concentration (SL1 and SL2)
Csol	Solvent Concentration (SL3)
Tox	Toxic Concentration (SL)
Eff	Chemical Oxygen Demand Removal Efficiency
pH	pH
VSS	Effluent Volatile Suspended Solids
Physiological Data:	
SAA	Specific Acetoclastic Activity
SHMA	Specific Hydrogenotrophic Methanogenic Activity
Morphological Data:	
LfA	Total Filaments Length per Total Aggregates Projected Area
TL/VSS	Total Filaments Length per Volatile Suspended Solids
VSS/TA	VSS per Total Aggregates Projected Area (Apparent Granules Density)
>1	Percentage of Aggregates Projected Area with Equivalent Diameter (D_{eq}) ≥ 1 mm
>0.1	Percentage of Aggregates Projected Area within the range 0.1 ≤ D_{eq} (mm) < 1 mm
<0.1	Percentage of Aggregates Projected Area with D_{eq} < 0.1 mm
Vsed	Settling Velocity (SL)

7.3 RESULTS AND DISCUSSION

7.3.1 Organic Load Disturbances

7.3.1.1 Recognition of load disturbances effects

The extraction of three Principal Components (PCs) gathered more than 80% of the total variability in all four datasets (Table 7.2). The use of other PCs did not enhance significantly the correlation factors.

Table 7.2 – Total variability in datasets, contained in the first three Principal Components.

Principal Components	LD1	LD2	LD3	LD4
PC1	44.4 %	44.6 %	56.6 %	50.6 %
PC2	20.5 %	22.5 %	27.4 %	27.4 %
PC3	19.3 %	18.9 %	14.5 %	14.5 %
Cumulative	84.2 %	86.0 %	92.5 %	92.5 %

The projection of an observation/sample on a PC is called the score of the observation on that PC. The score maps (Figure 7.1) can be seen as windows in the X space, displaying the observations as situated on the projection planes or hyperplanes, and revealing groups, trends, outliers and similarities between samples.

PC1–PC2 plane (Figure 7.1) encloses the most information of all planes that can be drawn through the data in the n-dimensional space. Two clusters clearly isolate the observations during the load disturbances and the recovery period, besides the inoculum sample, which emerge as an isolated observation. This shows that immediately after the load disturbance, a deviation occurred.

The information that can be obtained about the variables and the role they play in the PC, resides in the so called loadings and respective loading maps (Figure 7.2). It allows deciding which variables are most important for the differences observed between the samples. The interpretation of the loadings is essentially done by looking at what variables have the higher coefficients (positive or negative) on a certain PC and are, therefore, more important for the scores on that PC. The loading plots can be very useful to detect correlations between variables. Variables close to each other, *i.e.* with similar coordinates, represent strongly correlated variables. Concurrently, variables with symmetric coordinates are correlated, although inversely proportional.

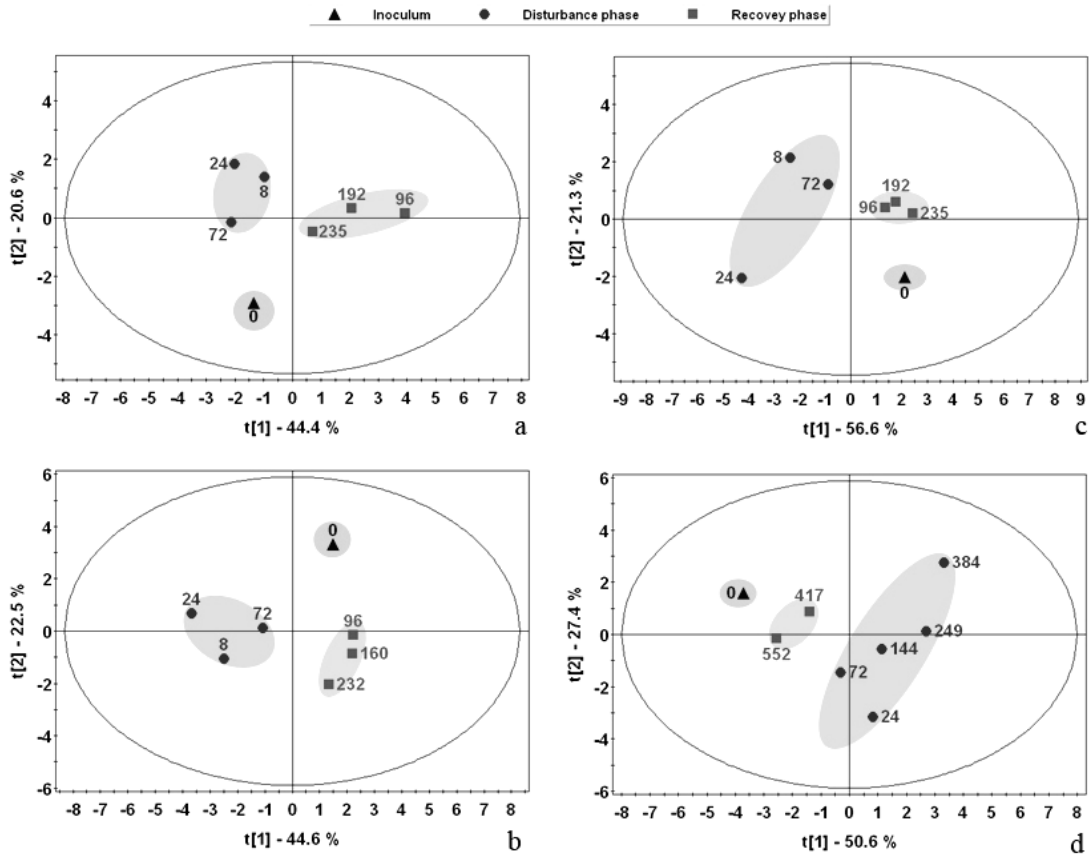

Figure 7.1 – PCA score plot of the first PC (t[1]) versus the second PC (t[2]), in dataset of: (**a**) load disturbance 1; (**b**) load disturbance 2; (**c**) load disturbance 3; and, (**d**) load disturbance 4.

Combination of score and loading maps (Figure 7.1 and Figure 7.2) allows for the visualization of the main effects/problems occurred during EGSB reactors operation. For instance, the inoculum sample is located in the upper part of the plot, with high score in PC2 (Figure 7.1b, t[2]). The variables that most influence this PC are >1, >0.1, SHMA, Eff, and VSS (Figure 7.2b, p[2]) that evidenced immediate changes when the disturbance was applied. The main clusters refer to observations with negative (disturbance) and positive (recovery) scores in PC1 (Figure 7.1b – t[1]). The variables with higher loadings in PC1 (Figure 7.2b, p[1]), *i.e.* responsible for grouping disturbance and recovery phase observations are HRT, pH, SAA, OLR, LfA and TL/VSS. The LfA showed the highest variation caused by the HRT, i.e. the highest loading (Figure 7.2b, p[1]).

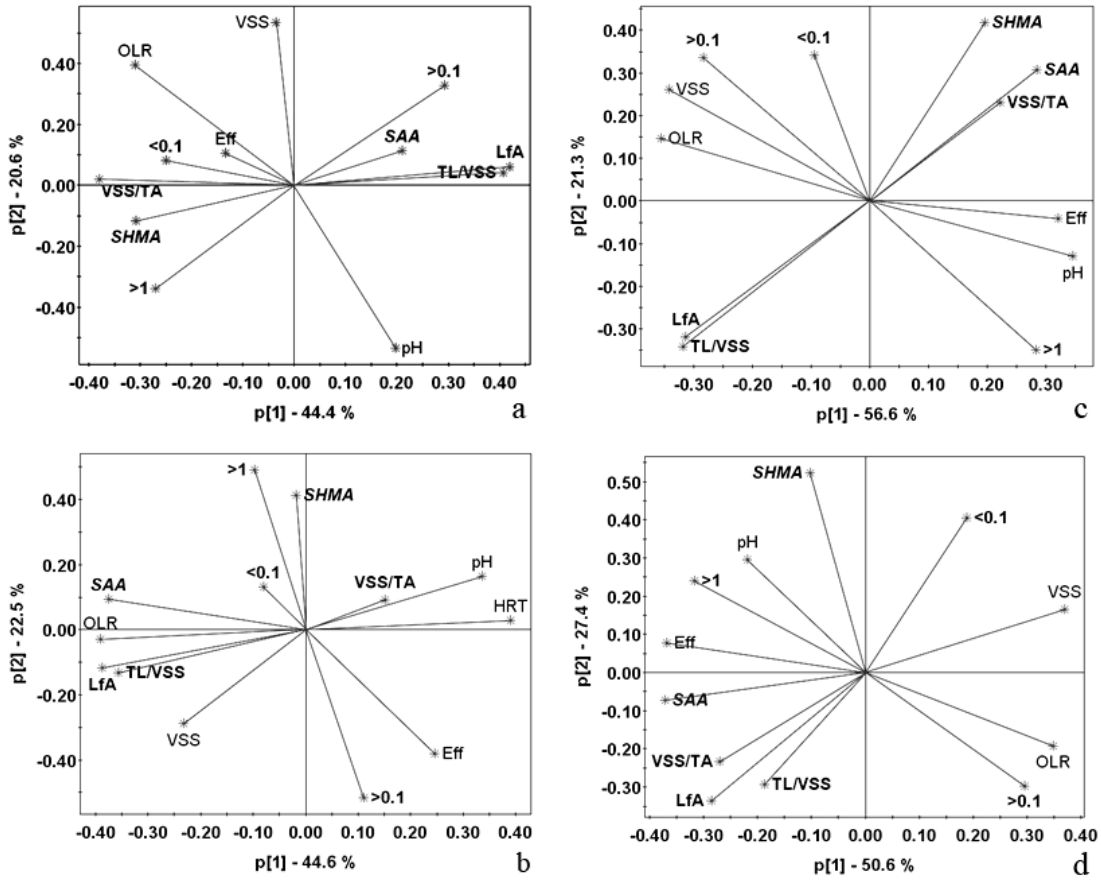

Figure 7.2 – PCA loading plot of the first and second principal components (p[1] vs. p[2]), from dataset of: (**a**) load disturbance 1; (**b**) load disturbance 2; (**c**) load disturbance 3; and, (**d**) load disturbance 4.

Observing LD3 (Figure 7.2c), is recognizable that the increase of OLR will cause the decrease of COD removal efficiency and pH, and the increase of granules fragmentation/erosion (decreasing the % of granules projected area with D_{eq}>1mm), effluent VSS, and % of granules projected area with D_{eq} < 0.1 mm. Other relations can be drawn from the loading maps of other load disturbances (Figure 7.2).

Some correlations should be distinguished from the loading maps. It was observed a high positive correlation between LfA and TL/VSS parameters in all datasets. Previously some hypotheses were postulated, regarding correlations between reactor performance deterioration, biomass activity, and erosion/fragmentation of

granules and consequent filaments release and washout. Emphasis should be given to the correlation showed in LD3 (Figure 7.2c) between apparent granules density (VSS/TA) and specific hydrogenotrophic methanogenic activity (SHMA), supporting the hypothesis presented earlier, where densification of aggregates was simultaneous with an increase in the SHMA (Abreu et al., 2007).

Special cares should be put in the analysis of the loading plot of LD4 (Figure 7.2d), because extending the period of disturbance, induced a new dynamic of filaments release and washout. Consequently, the correlations detected were poorer in this case. To elucidate this new dynamic, in Figure 7.2d, is observed a negative correlation between efficiency (Eff) and the % of aggregates with equivalent diameter within the range 0.1-1mm (>0.1). The increase of smaller aggregates suggests the occurrence of erosion and/or granules fragmentation. This phenomenon could have destroyed the granules structure and architecture, likely, exposing the inner core of acetoclastic bacteria to the bulk conditions. The decrease in the removal efficiency was probably associated to the selective washout of those bacteria, evidenced by the negative correlation between the SAA and the effluent VSS.

The increasing number of reports with methods for monitoring and control of anaerobic wastewater treatment processes does not consider the integration of quantitative morphological indicators. In the present work, the application of PCA to datasets obtained during load disturbances, allowed the establishment of a new latent variable that encompasses a weighted sum of performance, physiological and morphological information. This new variable can be used as a warning indicator of operational problems during load disturbances as can be clearly observed in Table 7.3. The variable t[1] was calculated in the first hours of operation in the load load disturbance and the corresponding percentage of variation in t[1] ranged between 27 and 268%, showing the high sensitivity of the latent variable to detect the disturbance. In LD1, the percentage of variation after eight hours was the smallest one (27%) because this was the softer shock applied with less significant effects.

Table 7.3 – Coefficients of the first latent variable t[1] in PC1, at the beginning and after few hours of load disturbance.

Load Disturbance	t = 0 h	t = 8 h	t = 24 h	% of variance
LD1	-1.343	-0.976	-	27 %
LD2	+1.482	-2.493	-	268 %
LD4	+2.132	-2.364	-	211 %
LD4	-3.701	-	0.817	122 %

For a better understanding of principal components analysis and the factors involved, is necessary to remember that the score (t_i) of an observation (i) on a principal component PC_j (t_iPC_j) is the weighted sum of the original variables (x_i). The weights (p_iPC_j) are called the loadings of the variables on that PC_j. The loading of a variable is related to its variation (Massart and Vander Heyden, 2005).

$$t_i PC_j = \sum p_i \, PC_j x_i \tag{7.1}$$

Analyzing the loadings associated with the new latent variable t[1] described previously it is possible to distinguish the variables that mostly influence the early detection of reactor disturbance. The morphological parameters, with emphasis to the LfA parameter that relates the free filaments length per aggregates projected area emerges due to its high loadings, i.e. weights (absolute value) in all datasets (Table 7.4). The results clearly suggest that quantitative morphological parameters should be considered in monitoring high rate anaerobic reactors, especially those based on granular sludge. It is important to mention, in this respect, that granular sludge is a highly valuable resource used to start-up industrial Up-Flow Anaerobic Sludge Blanket (UASB) reactors, Expanded Granular Sludge Bed (EGSB) reactors or Internal Circulation (IC) reactors. If available, cost of granular sludge can attain about 300€/ton, including 2000 km transportation. Therefore, physiological quality

but also morphological stability should be assessed when operating in a different reactor or in different conditions.

Table 7.4 – Loadings/weights of the variables in datasets associated to PC1.

Variable	LD1	LD2	LD3	LD4
HRT	-	0.390	-	-
OLR	-0.309	-0.390	-0.355	0.348
Eff	-0.134	0.245	0.320	-0.368
pH	0.197	0.336	0.345	-0.219
VSS	-0.036	-0.232	-0.341	0.369
<0.1	-0.250	-0.080	-0.094	0.189
>0.1	0.293	0.111	-0.283	0.295
>1	-0.270	-0.097	0.283	-0.316
SAA	0.211	-0.375	0.284	-0.372
SHMA	-0.308	-0.018	0.196	-0.102
LfA	**0.419**	**-0.387**	**-0.314**	**-0.284**
TL/VSS	-0.380	0.151	0.222	-0.270
VSS/TA	0.407	-0.356	-0.318	-0.186

The integration of morphological information quantified by image analysis, reactor performance, specific activity, and chemometric methods constitutes a package for advanced monitoring of anaerobic granular sludge bed reactors, which is being tested at industrial scale with promising results.

7.3.1.2 Differentiate the organic load disturbances

A dataset enclosing all data from organic load disturbances was created in order to analyse the efficacy of PCA to distinguish organic from hydraulic overloads. Analysing the PC1-PC2 plane (Figure 7.3a), is visible that different deviations

occurred from the inoculum samples according to the type of shock load applied. The hydraulic shock load (LD2) samples were grouped in a cluster located in the right side of the plane (line 1 in Figure 7.3a), influenced by the increase of TL/VSS and LfA and the decrease in SHMA (Figure 7.3b). The organic shock loads LD3 and LD4 samples diverged to the left side of the graph (line 2 in Figure 7.3a). This trend became more evident with the increase of exposure time in LD4. The variables >1, VSS and Eff show the highest loadings (Figure 7.3b) since the granules fragmentation and consequent washout and COD removal efficiency decrease were the most significant effects in these disturbances.

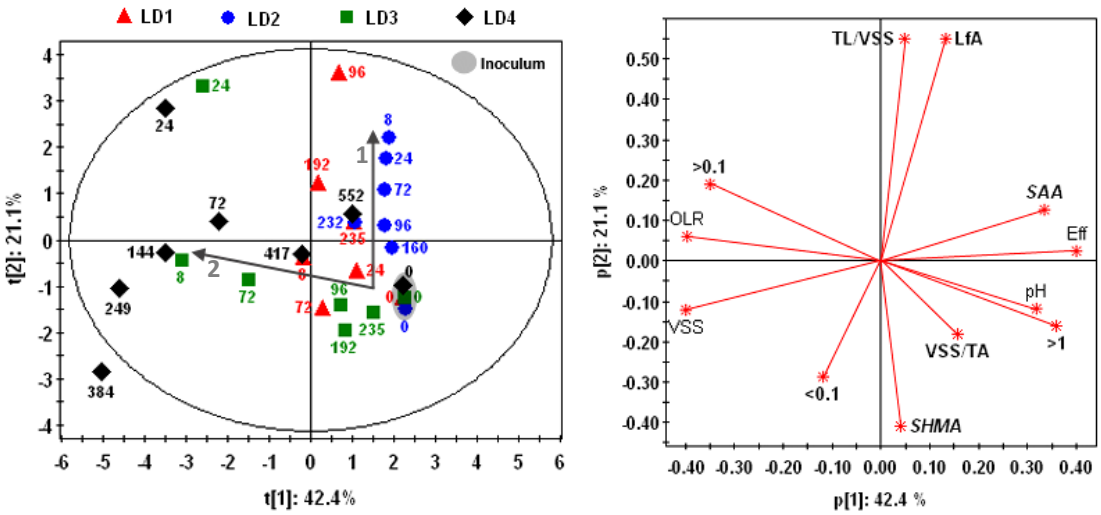

Figure 7.3 – PCA in dataset integrating data from all organic load disturbances: (**a**) score plot of the first PC (t[1]) versus the second PC (t[2]); and, (**b**) loading plot of the first and second principal components (p[1] vs. p[2]).

7.3.2 Toxic Shock Loads

7.3.2.1 Recognition of shock load effects

Applying a chemometric technique such as Principal Component Analysis (PCA) is advantageous when an effective reduction of the multi dimensional space into few

components is accomplished, while keeping the variability of the dataset. In this study, three principal components in detergent shock loads (SL1 and SL2) and four in solvent shock load (SL3) gathered more than 80% of the total variability in the datasets (Table 7.5).

Table 7.5 – Total variability in datasets contained in the first four Principal Components.

Principal Component	SL1	SL2	SL3
PC1	65.5 %	46.3 %	38.1 %
PC2	14.3 %	23.9 %	23.8 %
PC3	9.6 %	14.6 %	12.0 %
PC4	7.5	7.0 %	11.2 %
Cumulative	96.9 %	91.7 %	85.1 %

In the score plots of the first and second Principal Components, t[1] vs. t[2] (Figure 7.4a,c,e) is observed that samples were clustered regarding to its operational phase. A cluster encompassing the observations obtained during exposure phase is visible in each score plot. Besides, is clearly observed that a deviation occurred immediately after the shock loads were applied. The inoculum sample, which emerge as an isolated observation, is located far from the first observation during exposure time (see line in Figure 7.4a,c,e).

The influence that each measured variable had in each score, is given by its loadings, i.e. weighted variables, and respective loading maps (Figure 7.4b,d,e). Coupled visualization of score and loading plots (Figure 7.4) allows for the detection of the main effects/problems occurred during the shock loads. For instance, the main effects caused by SL1 were detected in the morphological parameters. The

introduction of the toxic compound in the feeding caused an increase in LfA and TL/VSS parameters and decrease in VSS/TA (Figure 7.4b). These results suggest changes at the granules microstructure level with release of filaments and decrease of apparent density (VSS/TA). However, during reactors operation, the COD removal efficiency remained unaffected.

Increasing the detergent concentration (SL2) caused an immediate decrease in specific acetoclastic activity (SAA) and VSS/TA. Analysing Figure 7.4c is observed that the inoculum sample is located in the top of the graph with the higher score in PC2. Simultaneously, the variables with higher influence in PC2, were SAA and VSS/TA (Figure 7.4d, p[2]). PC1 distinguished samples during exposure time (positive scores) from samples during recovery phase/inoculum (negative scores) (Figure 7.4c, t[1]). Once more, the morphological parameters LfA and >1, were the most sensitive to recognize the shock load (Figure 7.4d, p[1]).

In SL3 the isolation of exposure phase samples is not so effective using only the first PC, since it gathered just 38.1% of the total dataset variability (Figure 7.4e, t[1]). However, considering the PC1–PC2 plane, a cluster encompassing these samples is visible. The granules size distribution (<0.1, >0.1 and >1) show high loadings in PC2 (Figure 7.4f, p[2]). Simultaneously, vsed and VSS present high loadings in PC1 (Figure 7.4f, p[1]). Therefore, it is possible to state that these were the variables with higher influence in the samples clustering. Thus, although the reactor performance deteriorates only in the last hours of the exposure phase, a change in the macrostructure of granules was observed immediately when the shock load was applied. In fact, the % of aggregates projected area with D_{eq} higher than 1 mm decreased from 81 to 53, indicator of granules fragmentation and consequent washout (increase of the effluent VSS).

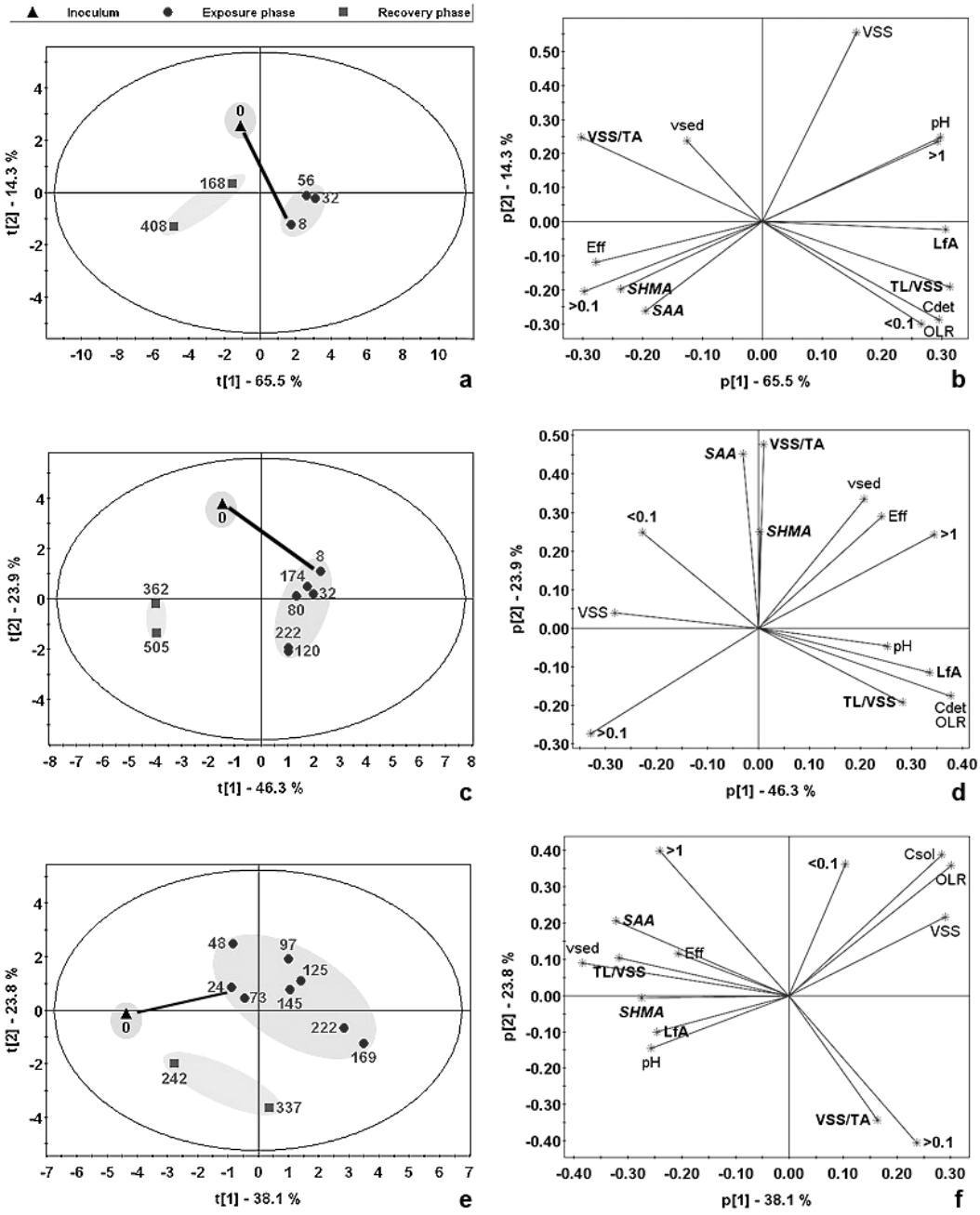

Figure 7.4 – PCA score plot of the first PC (t[1]) versus the second PC (t[2]), in dataset of: (**a**) SL1; (**c**) SL2; and, (**e**) SL3. And, PCA loading plot of the first and second principal components (p[1] vs. p[2]), from dataset of: (**b**) SL1; (**d**) SL2; and, (**f**) SL3.

In the last decade a vast number of methods to monitoring and/or control of wastewater anaerobic wastewater treatment technology have been proposed with different parameters as indicators of operational problems (Garcia et al., 2007, Lardon et al., 2005). The use of PCA illustrates the usefulness of monitoring the granules morphology to detect possible toxic contamination and future operational problems. The early detection of these problems is essential to attain timely control of the process before it evaluates to an irreversible problem. In this work was visible that morphological changes occurred before reactors performance deterioration, proving the sensitivity of the proposed parameters to detect the toxic events.

PCA provides information on the most meaningful parameters, which describes a whole dataset affording data reduction with minimum loss of original information (Helena et al., 2000). During PCA, it was defined a new latent variable, t[1], that includes a weighted sum of performance, physiological and morphological information. This new variable can be used as a warning indicator of operational problems during toxic shock loads. The variable t[1] was calculated for the inoculum and the first sample of exposure phase and the corresponding percentage of variation was 262, 254 and 80%, respectively in SL1, SL2 and SL3. As in organic loading disturbances, these results show the high sensitivity of the latent variable to recognize deviations of the normal process operation.

Analyzing the loadings/weights associated with the new latent variable t[1], it is possible to distinguish the variables that mostly influence the early detection of toxic contaminations. The morphological parameters emerge due to its high loadings in all datasets (Table 7.6). Once more, these results confirm that quantitative morphological parameters should be considered in monitoring and control of high rate anaerobic reactors, especially those based on granular sludge.

Table 7.6 – Loadings/weights of the variables in datasets associated to PC1.

Variable	SL1	SL2	SL3	Notes
Cdet	0.295	0.377	-	Controlled Variable
Csol	-	-	0.283	Controlled Variable
OLR	0.295	0.377	0.300	Controlled Variable
Eff	-0.278	0.241	-0.206	
pH	0.297	0.253	-0.256	
VSS	0.158	-0.280	0.290	
<0.1	0.265	-0.227	0.104	Morphological Variable
>0.1	-0.297	**-0.329**	0.236	Morphological Variable
>1	0.293	**0.345**	-0.241	Morphological Variable
SAA	-0.194	-0.030	**-0.321**	
SHMA	-0.236	0.003	-0.275	
LfA	**0.306**	**0.336**	-0.246	Morphological Variable
TL/VSS	**-0.302**	0.009	0.164	Morphological Variable
VSS/TA	**0.313**	0.283	**-0.316**	Morphological Variable
vsed	-0.126	0.207	**-0.384**	

7.3.2.2 Differentiate the toxic shock loads

A PCA in a dataset integrating all available information was performed in order to highlight differences between the shock loads. Watching at Figure 7.5a three clusters, one for each shock load, can be distinguished. The cluster encompassing the SL2 samples is isolated from the others. Effectively, SL2 caused the most negative effects to the anaerobic granular sludge, since it was the only one where the COD removal efficiency decreased significantly during the exposure phase.

The score and loading plots of PC1 and PC2, t[1] vs. t[2] and p[1] vs. p[2] (Figure 7.5), show the variables with higher influence in each shock load. SL1 was characterized by an increase in TL/VSS and LfA. The decrease of Efficiency (Eff) and SAA and increase of granules density describe SL2. Regarding to SL3 it was

categorized mostly by the granules D_{eq} ranges >1 and >0.1, sign of granules fragmentation.

Searching for possible correlations between variables, it is possible to observe a high positive correlation between the total filaments (TL/VSS) and the dynamic of filaments per area of aggregates (LfA) (Figure 7.5b). Therefore, can be hypothesized that the granules microstructure stabilization, by locking the filaments inside the aggregates, play a more important role in the maintenance of a high efficiency than granules macrostructure/size stabilization.

During the shock loads was observed that LfA increased 3, 5, and 2 days before effluent volatile suspended solids, respectively in SL1, SL2, and SL3. It was hypothesized that LfA could be an early-warning indicator of washout events (Amaral et al., 2004, Costa et al., 2007). In Figure 2b is visible that LfA and VSS are inversely proportional, enhancing the hypothesis that LfA increases before VSS, decreasing afterwards, when VSS increases.

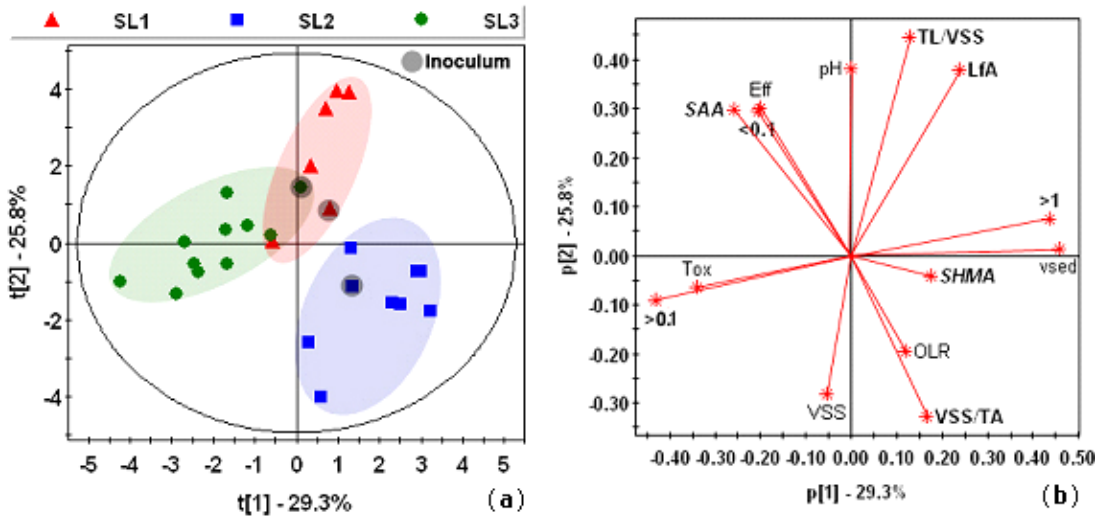

Figure 7.5 – PCA in dataset integrating data from all shock loads: (**a**) score plot of the first PC (t[1]) versus the second PC (t[2]); and, (**b**) loading plot of the first and second principal components (p[1] vs. p[2]).

7.3.3 Distinguish organic load disturbances from toxic shock load

With the aim of study the possibility of distinguish organic loading from toxic shock disturbances with Principal Components Analysis, a dataset gathering all information retrieved from experiments previously described (Chapters 4 to 6) was created.

Analyzing the score and loading plots of PC1-PC2 plane (Figure 7.6) is possible to visualize the variables with higher influence to distinguish load disturbances from shock load observations and respective effects, *i.e.* the variables more affected by the transient instabilities. The organic loading disturbances displaced the observations in direction of negative scores in PC1 and PC2 (line 1 in Figure 7.6a). Concerning to the toxic shock loads, it was observed that observations were dislocated in the direction of positive scores in PC1 and negative scores in PC2 (line 2 in Figure 7.6a).

Studying the direction of the lines from the exposure phase observations (Figure 7.6a) and the loading plot (Figure 7.6b), is perceptible that the toxic shock load affects mostly the morphological variables LfA and TL/VSS. Otherwise, the organic load disturbances caused increases in the VSS, and, decreases in the pH and COD removal efficiency. A severe fragmentation phenomenon was also observed, as showed by the increase in the percentage of aggregates projected area with equivalent diameter (D_{eq}) between 0.1–1 mm (variable >0.1), and consequent decrease in the % of aggregates area with Deq > 1 mm (variable >1). It should be emphasized that the observations from the recovery phases had tendency to return to close the inocula observations (Figure 7.6a), because the disturbances/shocks and respective effects were transient.

The results enhance the important role that biomass morphology monitoring can have in the recognition of organic loading and toxic disturbances in anaerobic granular sludge reactors. Indeed, the proposed morphological parameters were sensitive to detect the disturbances before COD removal efficiency decreased.

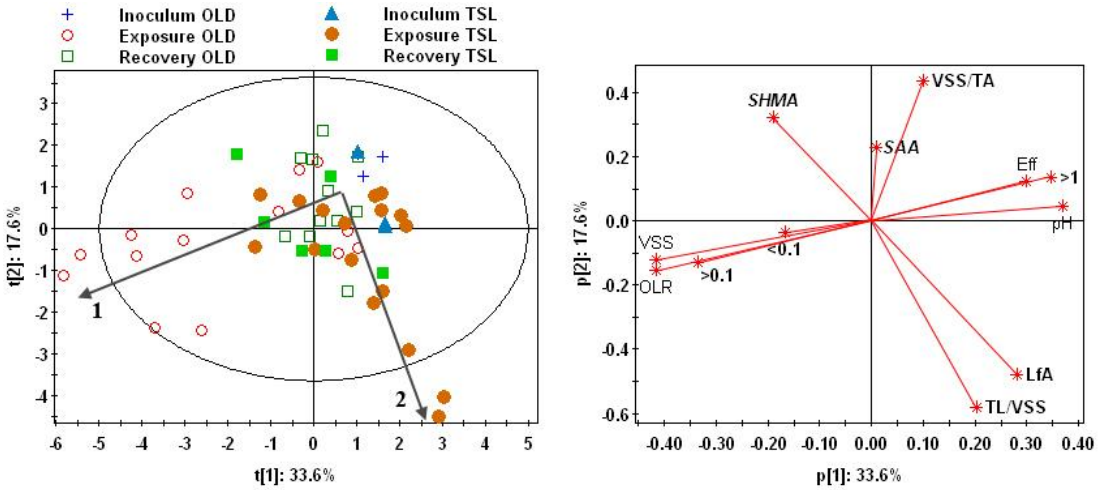

Figure 7.6 – PCA in dataset integrating all available data: (**a**) score plot of the first PC (t[1]) versus the second PC (t[2]); and, (**b**) loading plot of the first and second principal components (p[1] vs. p[2]).

7.4 CONCLUSIONS

Principal Component Analysis was performed in datasets gathering morphological, physiological and reactor performance information obtained during four organic load disturbances and three toxic shock loads. It was demonstrated that the use of a multivariate statistical tool such as Principal Components Analysis was appropriate to visualize and isolate the main effects caused by the transient instabilities.

The proposed morphological parameters proved to be more sensitive to detect promptly the problems than the normal operating parameters, such as Chemical Oxygen Demand removal efficiency.

The new latent variable t[1], defined as an weighted sum of all variables included in the dataset, showed high variations in the first hours of disturbance in all datasets. Concurrently, the high loadings/weights of the morphological parameters enhanced the need to monitor the anaerobic process solid phase in order to achieve an effective and feed forward control.

The PCA in datasets integrating information of all organic load disturbances and all toxic shock loads, proved to be appropriate to distinguish, not only the magnitude of the disturbance but also the nature of instability (organic overload or toxic).

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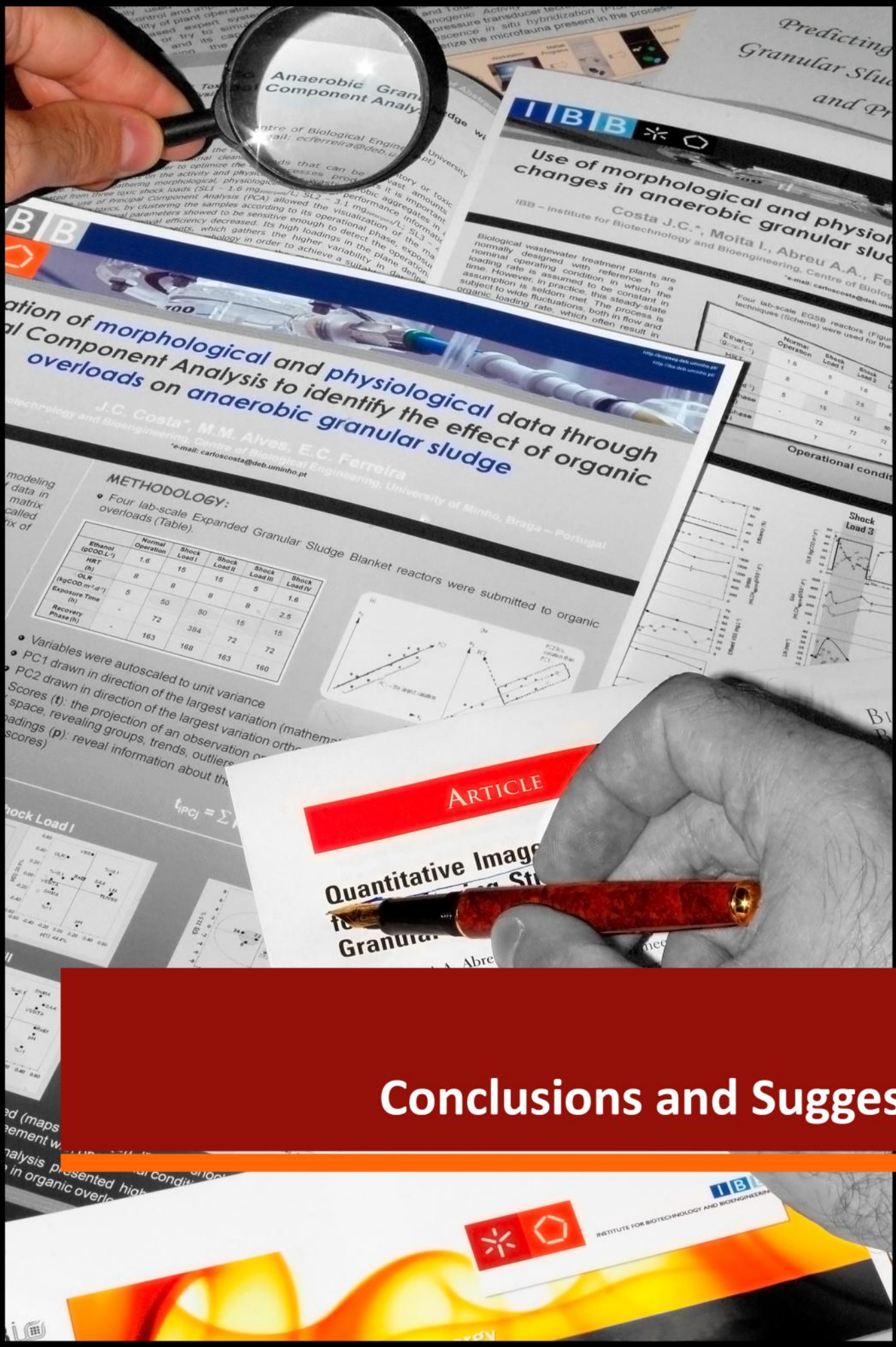

Conclusions and Suggestions

ABSTRACT

In this section, the main conclusions obtained within this work are presented. Also, some suggestions about future work in this field are leaved.

8.1 GENERAL CONCLUSIONS

The high efficiency of high-rate anaerobic reactors makes them an attractive technology for industrial wastewater treatment. Furthermore, a renewable energy source (biogas) is obtained during the process. Although all the advantages of this technology, the costs associated with granular sludge acquisition and replacement is one of the major drawback of the technology. This weakness became evident when some problems interrupt the normal operating state of the process, with consequent biomass disruption and washout. During this thesis, the effects of transient organic and toxic (detergent and solvent) load disturbances in the stability of anaerobic granular sludge were studied using quantitative image analysis. The importance of the proposed parameters was investigated using the chemometric technique Principal Components Analysis.

A transient increase in the organic loading rate will be translated in different effects depending of the magnitude and the nature of that increase, and the disturbance extent. A step increase from 5 to 18 $kg_{COD}·m^{-3}·d^{-1}$ did not affect the COD removal efficiency when the increase was due to an increase in influent ethanol concentration, but the reactor efficiency dropped from 94 to 72 % when it was applied by decreasing the hydraulic retention time. When the increase was as higher as 50 $kg_{COD}·m^{-3}·d^{-1}$, the efficiency was severely reduced to less than 30%. Also, in this situation, increasing the exposure time, caused acetogenesis inhibition. It should be emphasised that the results suggest that stability in the granules size distribution is of minor importance when compared to the capacity of filaments retention in the granular microbial structures. Effectively, only in the organic shocks

imposed by increasing the influent concentration, granules fragmentation/erosion was observed. In a hydraulic shock a negligible change in size distribution, due to erosion, was observed although an increase in TL/VSS was observed.

Concerning the toxic shock loads, the results obtained allow conclude that the inhibitory effect was dependent on detergent (surfactant) concentration and exposure time. The COD removal efficiency was unaffected with 150 $mg_{COD}·L^{-1}$ (56 hours exposure time) of detergent. However 80 h after exposure to 300 $mg_{COD}·L^{-1}$ of detergent, the COD removal efficiency decreased from 75 % to 17 %. It is relevant to note that in these conditions, the loss of performance was acute only after the complete inhibition of the specific acetoclastic activity. The overall reactor performance was unaffected by the presence of 40 $mg·L^{-1}$ of a solvent in the feeding during 222 hours.

Image analysis techniques allowed quantifying the structural stability of anaerobic granular sludge. Both detergent shocks induced an immediate increase of free filaments in the bulk medium, however, no significant size reduction was observed in the larger aggregates in both shocks. In solvent shock load, granules fragmentation/erosion was observed by the % of aggregates projected area with $D_{eq} \geq 1$ mm decrease from 81 to 53 %. The filaments length/total aggregate area (LfA) parameter confirmed to be sensitive to detect changes in the sludge morphology. LfA increased 3 and 5 days before the detection of a severe washout event, in detergent shock load I and II, respectively, suggesting that maybe it could be used as an early-warning indicator of biomass washout. Also, in solvent shock load, the peak in effluent VSS was observed 52 hours after LfA increased 35 %, highlighting the possibility of be used as an early warning indicator of biomass washout in the presence of toxic compounds.

During this thesis was demonstrated that the use of a multivariate statistical tool such as Principal Components Analysis was appropriate to visualize and isolate the main effects caused by the transient instabilities.

The proposed morphological parameters proved to be more sensitive to detect promptly the problems than the normal operating parameters, such as COD removal efficiency. The new latent variable t[1], defined as an weighted sum of all variables included in the dataset, showed high variations in the first hours of disturbance in all datasets. Concurrently, the high loadings/weights of the morphological parameters enhanced the need to monitor the anaerobic process solid phase in order to achieve an effective and feed forward control.

Results obtained within this work gave a better insight into the important role that monitoring the reactors solid phase (biomass), through quantitative parameters that describe the changes in granular sludge morphology, might have in the control of anaerobic wastewater treatment technology.

8.2 SUGGESTIONS FOR FUTURE WORK

The work described in this thesis adds significant insights into the importance of monitor the morphological changes in anaerobic granular sludge when several disturbances occur, in order to achieve a better control of the process. Two different lines of investigation should be considered to take real advantage of these techniques and definitively confirm their adequacy to control anaerobic wastewater treatment processes. Therefore, upcoming work needs to be focused on assessment in full-scale reactors, and, integration of the proposed parameters in a knowledge based system.

Future studies on this subject should be carried out in full-scale reactors. Indeed the uncertainty adjacent to real industry effluents is the ideal scenario to confirm the

usefulness, and test the response of these techniques to the wide variations in effluent flow rate and composition that are usually found.

The use of knowledge based expert systems (KBES) is a powerful tool to control anaerobic wastewater treatment reactors. The most used systems are based in artificial neural networks and fuzzy logic. While the firsts are appropriate when a large amount of data is available and when we have little knowledge about the plant behaviour, the second deals with the opposite problems, i.e. when a low amount of data is available and there is a good knowledge about the plant behaviour. In both cases the results obtained with the principal components analysis can be very usefulness as a preliminary step in development of a KBES. There are two possibilities for integration of image analysis results in a knowledge based expert system:

- The most simple and easier solution may be the use of the knowledge acquired during the last years with quantitative image analysis and respective recognition of structural changes occurred during process disturbances to define the if-then rules typical of a *fuzzy logic* system;
- Other possibility, and since a *neural network* system needs large amount of data to train and validate the system, the weights obtained in the principal components analysis can be used as a first attempt to train the network. Otherwise it is impossible to apply this system with morphological parameters due to the limited number of samples obtained.

Relatively to the image analysis technique, better results and correlations may be obtained if an effective distinguish between the free and protruding filaments can be obtained. This differentiation could be important in the early detection of washout phenomenon by a new parameter similar to LfA but that take in consideration only the protruding filaments. Effectively, this new parameter should increase before the free filaments detection in the bulk and its possible washout.

SCIENTIFIC OUTPUT

The overall work developed during the last four years of PhD grant give origin to the following publications:

PAPERS IN JOURNALS WITH PEER REVIEW:

1. Pires O.C., Palma C., **Costa J.C.**, Moita I., Alves M.M. & Ferreira E.C. (2006) Knowledge-based fuzzy system for diagnosis and control of an integrated biological wastewater treatment process. *Water Science and Technology* 53(4-5): 313-320.

2. Abreu A.A., **Costa J.C.**, Araya-Kroff P., Ferreira E.C. & Alves M.M. (2007) Quantitative image analysis as a diagnostic tool for identifying structural changes during a revival process of anaerobic granular sludge. *Water Research* 41(7): 1473-1480.

3. **Costa J.C.**, Abreu A.A., Ferreira E.C. & Alves M.M. (2007) Quantitative image analysis as a diagnostic tool for monitoring structural changes of anaerobic granular sludge during detergent shock loads. *Biotechnology and Bioengineering* 98(1): 60-68.

4. **Costa J.C.**, Moita I., Abreu A.A., Ferreira E.C. & Alves M.M. (2009) Advanced Monitoring of High Rate Anaerobic Reactors through Quantitative Image analysis of granular sludge and Multivariate Statistical Analysis. *Biotechnology and Bioengineering* 102 (2):445-456.

5. **Costa J.C.**, Alves M.M & Ferreira E.C. (2009) Principal Component Analysis and quantitative image analysis to predict effects of toxics in anaerobic granular sludge. *Bioresource Technology* 100 (3): 1180-1185.

6. Abreu A.A., Danko A.S, **Costa, J.C.**, Ferreira, E.C. & Alves, M.M. (2009) Effect of inoculum source and initial pH on bio-hydrogen production from L-arabinose a component of hemicellulosic biopolymers. *International Journal of Hydrogen Energy*, doi:10.1016/j.ijhydene.2008.12.020.

7. **Costa J.C.**, Moita I., Ferreira E.C. & Alves M.M. (2008) Morphology and physiology of anaerobic granular sludge exposed to an organic solvent. *Journal of Hazardous Materials* (in press).

8. **Costa J.C.**, Alves M.M. & Ferreira E.C. (2009) A chemometric tool to monitor high-rate anaerobic granular sludge reactors during load and toxic disturbances. *Biochemical Engineering Journal*, doi:10.1016/j.bej.2008.12.006.

PUBLICATIONS IN CONFERENCE PROCEEDINGS (with peer review):

1. Pires O.C., Palma C., **Costa J.C.**, Moita I., Alves M.M. & Ferreira, E.C. (2005) Knowledge-based fuzzy system for diagnosis and control of an integrated biological wastewater treatment process.

Proceedings of the 2nd IWA Conference on Instrumentation, Control and Automation, Busan, Korea, May 29 - June 2, Vol. 1, 247-255.

2. Pires O.C., Palma C., Moita I., **Costa J.C.**, Alves M.M. & Ferreira E.C. (2005) A fuzzy-logic based expert system for diagnosis and control of an integrated wastewater treatment. *Proceedings of the 2nd Mercosur Congress on Chemical Engineering – ENPROMER and 4th Mercosur Congress on Process Systems Engineering* (ISBN: 85-7650-043-4), Rio de Janeiro, Brasil, 8 pp. (in CD-ROM).

3. **Costa J.C.**, Moita I., Ferreira E.C. & Alves M.M. (2007) Morphology and physiology of anaerobic granular sludge exposed to organic solvents. *Proceedings of the 11th IWA World Congress on Anaerobic Digestion*, 23-27 September, Brisbane, Australia, 6 pp (in CD-ROM).

4. **Costa J.C.**, Alves, M.M. & Ferreira, E.C. (2007) Integration of morphological and physiological data through Principal Component Analysis to identify the effect of organic overloads on anaerobic granular sludge. *Proceedings of the 11th IWA World Congress on Anaerobic Digestion*, 23-27 September, Brisbane, Australia, 6 pp (in CD-ROM).

5. **Costa J.C.**, Alves, M.M. & Ferreira, E.C. (2008) Predicting effects of Toxic Events to Anaerobic Granular Sludge with Quantitative Image Analysis and Principal Component Analysis. *Proceedings CD of the International Symposium on Sanitary and Environmental – SIDISA 08*, 24-27 June, Florence, Italy, 8 pp.

6. **Costa J.C.**, Alves, M.M. & Ferreira, E.C. (2008) A chemometric tool to monitor high-rate anaerobic granular sludge reactors during load and toxic disturbances. *Proceedings of the 10th International Chemical and Biological Engineering Conference - CHEMPOR 2008*, 4-6 September, Braga, Portugal, 6pp.

ABSTRACTS & POSTERS IN CONFERENCES

1. Abreu A.A., **Costa J.C.**, Araya-Kroff P., Ferreira E.C., Alves M.M. (2005) Recovery of acetoclastic activity in anaerobic granular sludge, monitored by methanogenic activity measurements and image analysis. *Livro de Actas do Congresso MICRO'05-BIOTEC'05*, Póvoa de Varzim, 30 de Novembro a 3 de Dezembro, P198, p. 272.

2. **Costa J.C.**, Abreu A.A., Ferreira E.C., Alves M.M. (2006) Effects of detergent shock loads on anaerobic granular sludge morphology and methanogenic activity. *International Symposium on Environmental Biotechnology 2006 – Book of Abstracts*, Leipzig, Germany, p. 288.

3. **Costa J.C.**, Moita I., Abreu A.A., Ferreira E.C., Alves M.A (2007) Use of morphological and physiological information to identify changes in anaerobic granular sludge during organic overloads. *Livro de Actas do Congresso Micro '07 - Biotec '07 - XXXIII JPG*, Lisboa 30.11 - 2.12 2007, p. 151.

4. Abreu A.A, Danko A.S., **Costa J.C.**, Alves M.M. (2008). Effect of pH on fermentative hydrogen production from L-arabinose using mixed cultures. *Proceedings CD of the International Conference and Exhibition on Bioenergy.* Guimarães, Portugal, April 6th - 9th, 2 pp.