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Hospital wastewaters treatment: upgrading water systems plans and impact on purifying biomass

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Thèse de doctorat

"With A Valiant Heart, Nothing Is Impossible"

Jacques Cœur (vers 1400-1461)

To my parents
To flowers of my life: Hadil, Asmaa, Alnabil- Bachar and Ahmad
To my beloved wife

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List of publications

1. **Alrhmoun M.,** Casellas M., Dagot C: Evaluation of the Extracellular Polymeric Substances (EPS) by Confocal laser scanning microscopy in Conventional Activated Sludge (CAS) and advanced membrane bioreactor (MBR) treating hospital wastewater. ***Water Science and Technology**, 2014- 69.11 (2287-2294).*

2. **Alrhmoun M.,** Carrion C., Casellas M., Dagot C: Upgrading the performances of ultrafiltration membrane system coupled with activated Sludge reactor by addition of biofilm supports for the treatment of hospital effluents. ***Chemical Engineering Journal. Accepted in 20-9-2014.***

3. Stalder T., **Alrhmoun M**., Louvet J.N., Casellas M., Maftah C., Carrion C., Pons M.N., Ploy M.C., Dagot C. Dynamic assessment of the floc morphology, the bacterial diversity and a specific bacterial genetic support constitutive of an activated sludge processing an hospital effluent. ***Environment Science and technology**, 2013- 47(7909-7917).*

4. **Alrhmoun M.,** Casellas M., Baudu M., Dagot C: Efficiency of modified granular activated carbon coupled with membrane bioreactor for trace organic contaminants removal. *International **Journal of Chemical, Nuclear, Metallurgical and Materials Engineering** Vol:8 No:1, 2014*

5. **Alrhmoun M.,** Carrion C., Casellas M., Dagot C: Impact of hospital effluents on the EPS in submerged membrane bioreactor (MBR) and conventional activated sludge treatment. ***Bioresource Technology Journal**. Written to be submitted*.

6. **Alrhmoun M.,** Carrion C., Casellas M., Dagot C: Application of membrane biofilm bioreactor (MBBR) for hospital wastewater treatment: Performances and efficiency for organic micropollutant elimination. *Written to be submitted.*

7. **Alrhmoun M.,** Casellas M., Dagot C: Effect of PAC addition on UF-AS process for hospital wastewater treatment. *Written to be submitted.*

8. **Alrhmoun M.,** Maftah C., Casellas M., Dagot C: Multi- level Approach for the integrated assessment of bacterial distribution and their integron in different systems for treating hospital wastewater. *Written to be submitted.*

2011

1. <u>Alrhmoun M.,</u> Stalder T., Barraoud O., Casellas M., Ploy Marie-Cécile., Dagot C.: Fate of amoxicillin on an activated sludge system. **Colloque SFGP 2011 XIII** Congress of the French Society of Process Engineering**, Lille- grand palais from 29 November to 1 December 2011. Oral presentation. (Article)**

2012

2. <u>Alrhmoun M.,</u> Louvet J N., Stalder T., Maftah C., Pons M. N., Casellas M., Dagot C.: Treatment at the source of hospital wastewater activated sludge: feasibility and impact on biomass. **SFGP 2012** the French Society of Process Engineering**, Nantes 1- 2 Feb. 2012. Oral presentation. (Article)**

3. <u>Alrhmoun M.,</u> Louvet J N., Stalder T., Pons M. N., Casellas M., Dagot C.: Effects of hospital effluents in activated sludge biomass on: structures and sustainability. **Congress APTEN, Poitiers du 25 au 28 September 2012. Oral presentation. (Article)**

4. <u>Alrhmoun M.,</u> Casellas M., Dagot C.: Comparison of hospital effluent treatment in a conventional system (activated sludge) and a BRM: purification performance, polymer structure and extracellaires mud. L'EAU, objectif 2025 : ressources, usages, solutions. **Le 9ème Congrès International (GRUTTEE), Aix- en- Provence du 29 au 31 octobre 2012. Oral presentation. (Long abstract)**

2013

5. <u>Alrhmoun M.,</u> Casellas M., Dagot C.: A performance evolution of suspended and attached growth MBR systems in treating Hospital wastewater**. Membrane conference technology, Texas, USA from 25 to 28 Feb. 2013. Poster.**

6. <u>Alrhmoun M.,</u> Casellas M., Dagot C: Application of support media for the best biological nutrient removal in au submerged mbr for treating the hospital effluent. Congress symposium biofouling membrane processes: characterization, anticipation, control from 28 to 29 -5- 2013, **Faculty of Science and Technology Limoges, France. Poster. (Abstract)**

7. <u>Alrhmoun M.,</u> Casellas M., Dagot C.: TREATMENT at source hospital wastewater by extern membrane bioreactor: performance and impact on the biomasses. **PLUMEE 2013, Bacau, Romania. Oral presentation. (Article)**

8. <u>Alrhmoun M.,</u> Casellas M., Dagot C: Evaluation of the Extracellular Polymeric Substances (EPS) by Confocal laser scanning microscopy in Conventional Activated Sludge (CAS) and advanced membrane bioreactor (MBR) treating hospital wastewater, 7th IWA Specialised **Membrane Technology Conference and Exhibition for Water and Wastewater Treatment and Reuse 24-29 August 2013, Toronto, Canada. Oral presentation. (Article)**

9. <u>Alrhmoun M.,</u> Casellas M., Dagot C.: Performance comparison between suspended and attached growth MBR systems in treating hospitals wastewater. **The XIVeme Congress SFGP, (Lyon) from 8 to 10 October 2013. Oral presentation. (Article)**

10. <u>Alrhmoun M., C</u>asellas M., Dagot C: Morphological and biochemical characterization biofilm fixed on media supports in extern membrane bioreactor. Biofilm Congress 2013 from 19 to 21 November 2013, Pau, France. (Abstract)

11. <u>Alrhmoun M.,</u> Casellas M., Dagot C**:** Removal of trace organic contaminants in conventional and membranes bioreactors systems, **European chemistry meeting conference de 4 au 7 December 2013 en Montenegro.**

2014

12. <u>Alrhmoun M.,</u> Casellas M., Baudu M., Dagot C**:** Efficiency of modified granular activated carbon coupled with membrane bioreactor for trace organic contaminants removal, **ICCEBS 2014: International Conference on Chemical, Environmental and Biological Sciences, London 19-20 January 2014.**

13. *Alrhmoun M., Casellas M. , Dagot C:* the effect of support media on process performance and membrane fouling in submerged and extern membrane bioreactors treating hospital wastewater. **Membrane conference technology, Las Vegas, NV, USA from 10 to 13 March-2014. Poster. (Abstract+ Article)**

14. <u>Alrhmoun M.,</u> Casellas M., Dagot C: Hospital wastewater treatment by Membrane Bioreactor: Performance and Impact on the biomasses, IICBE in Dubai, 17-18 March 2014. **Oral Presentation.** (Article)

15. <u>Alrhmoun M.,</u> Casellas M., Dagot C: Efficiency of modified granular activated carbon coupled with membrane bioreactor for trace organic contaminants removal scientific day (GEIST), 5-September 2014. Oral presentation. (Abstract)

16. <u>Alrhmoun M.,</u> Casellas M., Dagot C: hospital wastewater treatment by activated sludge coupled with microfiltration: performance and impact on the biomasses. **The 10 Congress International (GRUTTEE), Limoges from 29 to 31 October 2012. Oral presentation.**

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LIST OF ABBREVIATIONS

WWTP   Wastewater treatment plants
UWWs   Urban Wastewaters
HWWs   Hospital Wastewaters
CAS   Conventional activated sludge
MBR   Membrane bioreactor
MBBR   Membrane biofilm bioreactor
AS- UF   Activated sludge- Ultrafiltration
MF   Microfiltration
UF   Ultrafiltration
GAS   Granular activated carbon
PAC   Powder activated carbon
COD   Chemical oxygen demand
BOD   Biological oxygen demand
TSS   Total suspended solids
VSS   Volatiles suspended solids
TN   Total nitrogen
SN   Soluble Nitrogen
EPS   Extracellular polymeric substances
PN   Proteins
PS   Polysaccharides
HA   Humic Acid - like substances
SMBR   Submerged Membrane bioreactor
EMBR   Extern membrane bioreactor
HRT   Hydraulic retention time
AS   Activated sludge
HE   Hospital effluent
SRT   Sludge retention time
NH4-N   Ammonium nitrogen
NO3—N   Nitrate nitrogen
NO2-N   Nitrite Nitrogen
TMP   Trans-membrane pressure
UV   Ultraviolet
PPCPs   Pharmaceutically active compounds and personal care   products
DO   Dissolved oxygen
BSA   Bovine Albumin Serum
AS   Activated Sludge
IF   Indic of Fluorescence
TOC   Total organic carbon

Log Kow — Octanol/ water partition coefficient
Log D — Octanol/ water partition coefficient
Kd — Sludge adsorption coefficient
pka — logarithmic constant of ionization
KPa — Kilo Pascal
L — Litter
M — Meter
Mg/L — Milligram par litter
Kg — Kilogram
Rc — Cake resistance
Rm — Intrinsic membrane resistance
Rf — Fouling Resistance
Rt — Total resistance
rpm — Rotations par minute
RNA — Ribonucleic Acid
s — second
SVI — Sludge Volume Index
ΔP — Transmembrane pressure
μ — Viscosity
μm — Micrometer
V — Volume of reactor
J — Permeate flux
Jc — Critical flux
EDCs — Endocrine Disrupting Compounds
y — Year
h — habitant
ng — nanogram
LC50 — Limit of Ecotoxicity
EC50 — Ecotoxicity
H — Hennery coefficient
Q air — Flow of air
C — Concentration
Ø — The fraction of compound volatilized
SS — Suspended solids concentration
K_{biol} — The degradation constant
FBR — Fluidized Bed Reactor
MLSS — Mixed Liquor Suspended Solids
POPs — Persistent Organic Pollutants
SAGB — Submerged Attached Growth Membrane
PVC — Polyvinyl Chloride

AEBR   Aerobic expended bed reactor
SAFF   Submerged Aerated Fixed Films
F/M   Ratio Food/ Microorganism
TMR   Tetraethyl rhodamine
TRITC   Isothiocyanate derivative
EEM   Extraction- Emission matrix
IR   Infra red
A   Absorbance
E   Molar absorptivity
LC/MS   Liquid Chromatography combined with mass spectrometry
GC   Gas Chromatography
GC/MC   Gas Chromatography- mass spectrometry
HPLC   High Performance Liquid Chromatography
UPLC   Ultra Performance Liquid Chromatography
HILIC   Hydrophilic Interaction Liquid Chromatography
NPLC   Normal Phase Chromatography
RPLC   Reversed- Phase Liquid Chromatography.
MS   Mass Spectrometry
LOQ   Lower limits of Quantitation
UE   Urban Effluents
d   day
°C   Celsius
mm   millimetre
IM   Indication Molhman
IB   Indication of sludge
DNA   Deoxyribonucleic acid
PCR   Polymerase Chain Reaction
DGGE   Denaturing Gradient Gel Electrophoresis
qPCR   Quantative PCR
R   Reactor
SPE   Liquid- Solid Phase
CLSM   Confocal Laser Scanning Microscopy
AG-MBR   Attached Growth Membrane Bioreactor
SG-MBR   Suspended Growth Membrane Bioreactor
ARD   Antibiotic resistance determinate
Qc   Quality Control
AMX   Amoxicillin
Mw   Molecular weight
pKd   -log 10[Kd]

INTRODUCTION

➢ Foreword

This is a thesis on organic micropollutants in the aquatic environment. At the beginning of such a thesis it may be adequate to deal with some basic questions regarding the reason why I have spend three years of time to study organic micropollutants in the environment and specially in the hospital wastewater and technologic treatment systems for the wastewater ! I think you may have a quick answer to such a question: "We as scientists care for the environment, and micropollutants are a threat."

Yet, this statement provokes the next question: "How does the environment benefit if we study micropollutants in the aquatic environment and report on the results for the people without find new techniques or development of technical concepts to eliminate or avoid damage in the environment?

➢ Problem statement

There are 60 to 80 thousand chemicals in regular use entering millions of different "environmental systems" (lakes, rivers, groundwater, soil, organisms).

In 2005, EPA began studying environmental contamination by pharmaceuticals, detergents, natural and synthetic hormones, and other chemicals. These contaminants are commonly referred to collectively as contaminants of emerging concern.

Many organic micropollutants are believed to enter municipal wastewater through numerous industrial, commercial and domestic applications (bathing, cleaning, laundry, and the disposal of unused pharmaceuticals and human waste).

Normally, the hospital wastewaters (HWWs) are assimilated to urban wastewaters (UWWs) in many countries where they are discharged into municipal sewage and collected to a wastewater treatment plant (WWTP) where they are co-treated with urban or/and industrial effluents. This practice, considers that hospital and urban wastewaters are similar in terms

of pollutants, concentrations and loads. Decidedly, this is not a correct assumption, because these HWWs are really different. As a result, the collection of hospital wastewater together with domestic wastewater has been criticised and a dedicated pre-treatment of hospital wastewater has been recommended (Verlicchi *et al.,* 2010; Gupta *et al.,* 2009; Pauwels and Verstraete, 2006).

Persistent substances may pass the wastewater treatment plant (WWTP) unchanged. In addition, input of easy degradable substances occurs through WWTPs that are not state of the art and periodically through storm water or combined sewer overflows. If several WWTPs drain into the same water body, micropollutants can accumulate along the stretch or in lakes. Even groundwater used as drinking water may be contaminated by micropollutants from urban drainage via infiltration of polluted surface water.

Micropollutants may have adverse effects on aquatic life even at very low concentrations.

Usage, physical-chemical and ecotoxicological properties determine whether a substance causes problems in the aquatic environment. The concentration of a compound in the WWTP effluent is determined by the load into the wastewater treatment plant and the physico-chemical properties of the compound. Generally, substances that are water soluble and persistent are not removed in WWTPs and can therefore be detected in natural waters. High concentrations occur principally in small streams with a high fraction of treated wastewater. The comparison of the exposure with ecotoxicologically based thresholds allows to asses the risk to affect the aquatic life. In this detail we can really estimate the importance to research for another technology more effective as, by example, the membrane bioreactor (MBR).

The membrane bioreactor (MBR) for wastewater treatment has been currently one of the new technologies for both municipal and industrial wastewater treatments, especially when the effluent is intended for water reuse (Chang *et al.,* 2002).

The membrane bioreactor technology has been available for around 40 years. But only the last 20 years that has seen a rapid growth on its implementation and subsequent significant penetration of the municipal market, coinciding with the introduction of the submerged configuration (SMBR) (Judd and Judd, 2006; Yamamoto *et al.,* 1989).

The use of Membrane Bioreactors (MBR) in hospital wastewater treatment has grown widely in the past decades. The MBR technology combines conventional activated sludge treatment with low-pressure membrane filtration, thus eliminating the need for a clarifier or polishing filters. The membrane separation process provided a physical barrier to contain microorganisms and assures consistent high quality reuse water. Few studies was found in the literature explained the efficiency of MBR in treating the hospital wastewater and removal the pharmaceutical s compounds. The wastewater treatment technologies analyzed included microfiltration, ultrafiltration, Nanofiltration, granular activated carbon, powdered activated carbon, reverse osmosis, electro dialysis reversal, membrane bioreactors, and combinations of these technologies in series. But, the principal problem concern the membrane bioreactor was the fouling which decreased the performance and increased the economic cost. Many studies were attributed to numbers of parameters and important one was presence the extracellular polymeric substances (EPS) and fouling rate. (Bourgeous *et al.,* 2001; Bouhabila *et al.,* 2001; Nagaoka *et al.,* 1996). For that the EPS was important key for studding performance the MBR in treating the Hospital wastewater.

➢ Objectives of this study

Real conditions study for impact and effects the hospital effluents on the treatment systems process and the microorganisms in different technologies was required, in addition to improving the organic micropollutants removal process in membrane bioreactor technology.

In the present study, conventional activated sludge pilot and membrane bioreactor systems in submerged and extern membrane (SMBR, EMBR) was proposed for the simultaneous removal of organics micropollutants contained in hospital wastewater.
The performance of these laboratory-scales (CAS and hybrid MBR systems) was investigated. In addition, the membrane fouling phenomenon was also studied. The effects of hospital effluents on the sludge were tested in different technologies and confirmed by optical analyses instruments as confocal microscopy, Infra red and Fluorometry. The influence of Extracellulair polymeric (EPS) and their compounds as polysaccharides, humic substances-

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like and proteins on membrane fouling was also investigated. In additions to development the MBR technologies for reach a high removal for the organic micropollutants was required.

In summary, the objectives of the present study are:

1. To examine the effects of hospital wastewater on the treatment performance of the MBR and CAS systems.
2. To study the toxic impact of hospital effluent on the microorganisms and characterize the changes in composition the sludge to decrease the membrane fouling phenomenon under different reel operating conditions.
3. To develop a MBR system to achieve high removal of organic micropollutants in treating the hospital effluents and produce a high quality effluent in the outlet.

➢ Scope of this study

This research was conducted with a focus on the development of a MBR system for organic micropollutants removal from the hospital wastewater and on the investigation of the main effects in the sludge suspension that worsens the performance of membrane bioreactor and increasing the membrane fouling problem.

➢ Assessment of the state of the art

The introduction gives a brief description on the background and motivation of the research. This parte also points out the aims and objectives of the study. The scope of the research as well as the overall structure of the thesis is also outlined in these pages.

In Chapter 1, previous studies on hospital wastewater: presence in the environment, composition and groups, treatments systems process (CAS and MBR) and their capacity in removal the organics micropollutants in wastewater, development the MBR used for high removal in wastewater in addition to characterise the EPS compounds and their role in membrane fouling process and control are reviewed.

Chapter 2 describes the materials and methods used in this laboratory study, including the experimental setup, characterisation the reel influent wastewater, operating conditions, and analytical methods.

Chapter 3 present experimental results on:

1. Evaluation of the extracellular polymeric substances by confocal laser scanning microscopy in conventional activated sludge and advanced membrane bioreactors treating hospital wastewater (Article 1 published in Water Science and Technology, 2014).

2. Dynamic Assessment of the Floc Morphology, Bacterial Diversity, and Integron Content of an Activated Sludge Reactor Processing Hospital Effluent (Published in Environment Science and Technology, 2013).

3. Impact of hospital effluents on the EPS in submerged membrane bioreactor (MBR) and conventional activated sludge treatment (Accepted in Biocenology Advances)

4. Application of membrane biofilm bioreactor (MBBR) for hospital wastewater treatment: Performances and Efficiency for Organic Micropollutant Elimination (Accepted in Biorescource Technology)

5. Upgrading the performances of Ultrafiltration Membrane system coupled with Activated Sludge Reactor by addition of biofilm supports for the treatment of hospital effluents (Accepted in Chemical Engineering Journal, 2014l)

6. Efficiency of Modified Granular Activated Carbon Coupled with Membrane Bioreactor for Trace Organic Contaminants Removal (Published in International Journal of Chemical, Nuclear, Metallurgical and Materials Engineering, 2014).

7. Effect of PAC addition on UF-AS process for hospital wastewater treatment. (Written to be submitted).

8. Multi- level Approach for the integrated assessment of bacterial distribution and their integron in different systems for treating hospital wastewater. (Written to be submitted).

9. Appendix (A): Investigations of effects the amoxicillin on activated sludge, and on antibiotic resistance (Written to be submitted).

10. Appendix (B): Effect of internal heterogeneity of activated sludge flocs on sorption of antibiotics. CLSM study of preferential sorption of vancomycin on Gram+ bacteria (Written to be submitted).

Finally, the conclusion summarizes the work of this research on innovative organic micropollutants removal MBR system, reviews the achievements of the research objectives and highlights the main findings. It concludes by giving recommendations on potential areas for further research.

Table chronological of thesis

2011-2012	Octobre- Novembre 2011	Novembre- décembre 2011	Janvier- mars 2012

Article 2 :
Pilote : BA- Effluents : CHU+ domestique –
Objectifs : 1. Etude des effets des effluents hospitaliers sur le procédé et les boues activées. 2. Analyses microscopiques et statiques sur la composition des flocs dans les recteurs.
Prélèvement : CHU Limoges + assin-sement domestique de Limoges

Article 3 : Trois pilotes : Batch Réacteur+ BRM Immergée + BA- Effluents : KSO+ injection de solution Amoxicillin en 100µg/L–
Objectifs : 1. Etude des effets l'Amoxicillin sur fonctionnemennt des procédés et les boues activées
2. Performance des deux procédés à éliminer l'Amoxicillin
Prélèvement : KSO Limoges-environ 380 L / Semaine

Article 3 : Deux pilotes : BRM Immergé + BA- Effluents : CHU– Objectifs : 1. Etude des effets des effluents hospitaliers sur le MBR et les boues activées (EPS...)
2. Performance des deux procédés à éliminer les micropolluants organiques
3. Analyses microscopiques et statiques sur la composition des flocs dans les reacteurs. Prélèvement : CHU Limoges- environ 440 L / Semaine

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + Cône d'Imhoff (100 mL) +chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+..

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ PCR+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectromètre de mass (LC/MS) +Spectrofluorimétrie+ Ph métrie + test d'adsorption avec membrane

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocale+ PCR+ Lowry et al. (1951) modifié par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectrofluorimétrie

2011-2012	Mai- juin 2012	Juillet 2012	Novembre- 2012 au mars 2013

Article 4 :
Pilote : Deux pilotes : BRM Immergé + BA- avec couplage des biofilms sur supports Effluents : CHU
Objectifs : 1. Etudier le rôle efficace de culture fixée des biofilms sur traitement des effluents hospitaliers. 2. Analyses microscopiques et statiques sur la composition des flocs dans les réacteurs. 3. Etudier le changement et la distribution des EPS et le colmatage Prélèvement : CHU Limoges - environ 440L / Semaine

Article 8 :
Pilote : Deux pilotes : BRM Immergé + BA- avec couplage des biofilms supports Effluents : CHU
Objectifs : 1. Etudier la sorption de la vancomycine sur les flocs bactériens de boues activées
2. Analyses microscopiques et statiques sur la composition des flocs dans les réacteurs.

Article 5 :
Pilotes : BRM Externe –Taille 500L
Effluents : CHU
Objectifs : 1. Etudier l'efficacité du traitement des effluents hospitaliers, impact sur les biofilms et le changement et la distribution des EPS avec le colmatage

Prélèvement : CHU Limoges - environ 550L / Semaine

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectromètre de mass (LC/MS)

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL +Chromatographie liquide + Spectromètre de mass (LC/MS)

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectromètre de masse (LC/MS) +Spectrofluorimétrie

| 2012-2013 | Mars au mai 2013 | | Juin- juillet 2013 |

Article 5 :
Pilotes : BRM Externe+ biofilms sur supports–Taille 500L
Effluents : CHU
Objectifs : 1. Etudier le rôle de culture fixée sur le traitements des effluents hospitaliers.
, impact sur les biofilms et le changement et la distribution des EPS avec le colmatage – Mesurer les biomasses fixées sur les supports et faire une relation avec EPS et colmatage. Prélèvement : CHU Limoges - environ 550L / Semaine

Article 6 :
Pilotes : BRM Externe –Taille 500L+ Poste de traitement par Charbon actif en grain modifié (deux colonnes longueur : 75 cm et largeur 5 cm)
Effluents : CHU
Objectifs : 1. Etudier l'efficacité de traitement des effluents hospitaliers, impact sur les biofilms et changement, distribution des EPS avec le colmatage
2. Etudier efficacité le charbon actif modifié à éliminer les micropolluants organiques. Test d'adsorption- Analyses isométriques.
Prélèvement : CHU Limoges - environ 550L / Semaine

Article 7 :
Pilotes : BRM Externe –Taille 500L+ Poste de traitement par Charbon actif en poudre (réacteur séparé après le bioréacteur et avant la membrane externe)
Effluents : CHU
Objectifs : 1. Etudier l'efficacité de traitement des effluents hospitaliers, impact sur les biofilms et l changement, distribution des EPS avec colmatage
2. Etudier efficacité le charbon active en poudre à éliminer les micropolluants organiques. Test d'adsorption- Analyses isométriques. + teste du colmatage pour étudier rôle le charbon actif à diminuer ou augmenter le colmatage ;. **Prélèvement :** CHU Limoges 550L/S environ 550L /

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ micro - scopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chroma-tographie liquide + Spectromètre de masse (LC/MS) +Spectrofluorimétrie+ Mesurer le sépaisseurs des biofilms sur les supports (Microscopie fucales inverse avec logiciel …)

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ioniques + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectromètre de mass (LC/MS) +Spectrofluorimétrie+ Ph métrie..

Méthodes :
Colorimétrique Hach gamme 0-1500 mg O2/L + chromatographie ionique + microscopie optique + spectroscopie UV- visible+ microscopie confocal+ PCR+ Lowry et al. (1951) modifiée par Frølund et al. (1995)+ Dubois et al., 1956+ Cône d'Imhoff (100 mL+ Chromatographie liquide + Spectromètre de mass (LC/MS) +Spectrofluorimétrie+ Ph métrie..

Chapter I

Literature Review

1. Hospital Wastewaters

Hospital wastewater represents a particular type of effluent; this assumption has been often objected and rejected since 1980. Scientists as Pauwels and Verstraete, 2006) and analytical campaign have been demonstrated and confirmed that the hospital effluents presents really different qualitative and quantitative characteristics (Altin *et al.,* 2003; kosma *et al.,* 2010; Liu *et al.,* 2010; Verlicchi *et al.,* 2010a) in compared with the urban wastewater. Hospitals generate on average 750 L of wastewater by bed and by day so they are 2-5 times higher than urban flow rates, which refer to one inhabitant equivalent (typically included in the interval 120-250 L). These effluents are loaded with pathogenic microorganisms, pharmaceutical partially metabolized, radioactive elements and other toxic chemical substances. Moreover, in hospital effluents, conventional pollutant (Among them BOD5, COD, TSS) is in general higher than in urban wastewaters (UWWs) (Verlicchi *et al.,* 2010a). Altin *et al.,* 2003; Chiang *et al.,* 2003; Brown *et al.,* 2006; Pauwels *et al.,* 2006; Kajitvichyanukul and Suntronvipart, 2006; Gautam *et al.,* 2007; Machado *et al.,* 2007; Sarafraz *et al.,* 2007; Tsakona *et al.,* 2007; Verlicchi *et al.,* 2008; Mesdaghinia *et al.,* 2009) observed that in hospital wastewaters (HWWs) BOD5, COD and SS keep 2–3 times higher than in (UWWs). The averages values in HWWs and UWWs are in (table 1).

Table 1: Average values in HWWs and UWWs. (Verlicchi *et al.,* 2009)

Parameter	UWWs	HWWs	Ratio
BOD5 mg L^{-1}	90	200	2,2
COD mg L^{-1}	170	500	3
TSS mg L^{-1}	60	160	2,7

Normally, the hospital wastewaters (HWWs) are assimilated to urban wastewaters (UWWs) in many countries where they are discharged into municipal sewage and collected to a wastewater treatment plant (WWTP) where they are co-treated with urban or/and industrial effluents. This practice, considers that hospital and urban wastewaters are similar in terms of pollutants, concentrations and loads. Decidedly, this is not a correct assumption, because these HWWs are really different. As a result, the collection of hospital wastewater together with domestic wastewater has been criticised and a dedicated pre-treatment of hospital wastewater has been recommended (Verlicchi *et al.,* 2010; Pauwels and Verstraete, 2006). Indeed, more recently, with the development of sensitive analytical techniques, which make possible the detection of more and more active pharmaceutical compounds, it is now well

established that pharmaceuticals and their metabolites are present in the environment (Kümmerer, 2004b) with wastewater being the primary entry route. Sources include households' agriculture and pharmaceutical industries (Kümmerer, 2004b) and hospitals are often pointed out as a hot spot to pharmaceutical residues in influents of municipal wastewater treatment plant (WWTP) (Ternes *et al.,* 2006; Hawkshead, 2008). Hospital wastewaters mainly comprise products used in everyday life in large quantities, such as endocrine disrupting compounds (EDCs), pharmaceutical and personal care products (PPCPs), surfactants and surfactants residues, and various industrial additives.

1.1. Consumption of pharmaceuticals

Referring to pharmaceuticals, large amounts of different compounds are used worldwide and, in the last decade, their sales have been continuously increasing (Kummerer, 2001; Ternes and Joss, 2006; Jjemba, 2006; Lienert *et al.,* 2007b). In particular, the annual consumption of ibuprofren (an analgesic) was equal to 166 t year^{-1} in 1998 in France (population of 55.5 millions), 128 t year^{-1} in 2001 in Germany (population of 82.4 millions), 276 t year^{-1} in 2003 in Spain (population of 43.2 million), 180 t year^{-1} in 2001 in Canada (population of 30 millions); the annual consumption of sulphamehoxazole (an antibiotic) was equal to 22.4 t year^{-1} in France, 47 t year^{-1} in Germany, 12.7 t year^{-1} in Spain, the annual consumption of amoxicillin (another antibiotic) was equal to 110 t year^{-1} in 2001 in Germany as well as in Italy (population of 58 millions). In the USA, approximately 23,000 t antibiotics are used in total per year (Ternes and Joss, 2006). Thousands of pharmaceutical chemicals are in use today, particularly in developed countries (Rounds *et al.,* 2009); approximately 3000 to 4000 different pharmaceuticals ingredients are used in the EU today, including painkillers, antibiotics, blockers, contraceptives, lipid regulators, antidepressants, antineoplastics, tranquilizers, impotence drugs and cytostatic agents, (see table 2).

Table 2: Consumption of pharmaceuticals for European countries (Sheyla *et al.,* 2012)

Compound	Germany		Switzerland		France		Sweden	Spain
	(Kg y⁻¹)	(mg y⁻¹ inh⁻¹)	(Kg y⁻¹)	(mg y⁻¹ inh⁻¹)	(Kg y⁻¹)	(mg y⁻¹ inh⁻¹)	(mg y⁻¹ inh⁻¹)	(mg y⁻¹ inh⁻¹)
Acetaminophen	n.a.	n.a.	n.a.	n.a.	3, 303,077d	54, 389,5	n.a.	n.a.
Acetylsalicylic	n.a.	n.a.	n.a.	n.a.	396,212d	6524.2	n.a.	n.a.
Alprazolam	n.a.	n.a.	n.a.	n.a.	178d	2.9	n.a.	n.a.
Amoxicillin	n.a.	n.a.	n.a.	n.a.	333,233d	5487.0	n.a.	n.a.
Atorvastatin	n.a.	n.a.	n.a.	n.a.	7924d	130.5	n.a.	n.a.
Azithromycin	n.a.	n.a.	n.a.	n.a.	4073d	67.1	n.a.	n.a.
Bezafibrate	39,158e	475,2	1574e	215.6	20,852d	343.4	66.7	92.6
Bromazepam	n.a.	n.a.	n.a.	n.a.	2604d	42.9	n.a.	n.a.
Carbamazepine	83,299e	1010.9	6260e	857.5	33,364e	554.3	820.2	463.0
Clarithromycin	12,36	150.0	1700e	232.9	16,889e	276.1	n.a.	n.a.
Ciprofloxacin	n.a.	n.a.	n.a.	n.a.	12,186d	200.7	n.a.	n.a.
Cyclophosphamide	n.a.	n.a.	n.a.	n.a.	305.7f	4.9	n.a.	n.a.
Diclofenac	78,579	953.6	6819e	934.1	22,640e	370.1	375.9	747.7
Escitalopram	n.a.	n.a.	n.a.	n.a.	4.6d	0.08	n.a.	n.a.
Fluoxetine	n.a.	n.a.	n.a.	n.a.	3740d	61.6	n.a.	97.2
Flutamide	n.a.	n.a.	n.a.	n.a.	521f	8,3	n.a.	n.a.
Fluvoxamine	n.a.	n.a.	n.a.	n.a.	1121d	18,5	n.a.	n.a.
Gemcitabine	n.a.	n.a.	n.a.	n.a.	379.3f	6,1	n.a.	n.a.
Ibuprofen	250,792	3043.6	22,471e	3078,2	58,353e	953,8	7864.3	6391,2
Ifosfamide	n.a.	n.a.	n.a.	n.a.	121,4f	1,9	n.a.	n.a.
Iohexol	8053e	97,9	4614e	632,1	46,774e	764,5	n.a.	n.a.
Iopamidol	38,165	463.2	2739e	375.2	34,540e	564,6	n.a.	n.a.
Iopromide	97,817e	1187.1	8965e	1228.1	12,810e	209,4	n.a.	463
Levonorgestrel	n.a.	n.a.	n.a.	n.a.	90g	1.38	n.a.	n.a.
Lorazepam	n.a.	n.a.	n.a.	n.a.	585d	9.6	n.a.	n.a.
Mitomycin	n.a.	n.a.	n.a.	n.a.	3.01f	0.05	n.a.	n.a.
Naproxen	n.a.	n.a.	n.a.	n.a.	37,332d	614.7	n.a.	986.1
Omeprazole	n.a.	n.a.	n.a.	n.a.	8045d	132.5	n.a.	n.a.
Pantoprazole	n.a.	n.a.	n.a.	n.a.	5287d	87.1	n.a.	n.a.
Paroxetine	n.a.	n.a.	n.a.	n.a.	5515d	90.8	n.a.	n.a.
Progesterone	n.a.	n.a.	n.a.	n.a.	10,000g	153.7	n.a.	n.a.
Roxythromycin	7359e	89,3	149e	20.4	4182e	68.4	n.a.	9.3
Sertraline	n.a.	n.a.	n.a.	n.a.	6224d	102.5	n.a.	n.a.
Simvastatin	n.a.	n.a.	n.a.	n.a.	6943d	114.3	n.a.	n.a.
Sulfamethoxazole	53,600e	650.5	2300e	315.1	17,519e	286,4	160,4	294.0
Tamoxifen	n.a.	n.a.	n.a.	n.a.	377f	6	n.a.	n.a.
Trimethoprim	12,183e	147.8	520e	71.2	20,603e	336.8	n.a.	n.a.
17-αthynylestradiol	48.2b	0,58	3.96b	0,54	n.a.	n.a.	0.11	0.3

n.a.: Data not available.
b Data from (Carballa *et al.,* 2008) for 2005 in Sweden for 2001 in Germany, for 2000 in Switzerland and for 2003 in Spain.
c Data calculated in this study for Spanish population in January 2010: 47.02×106 inhabitants.
d Data from (Besse *et al.,* 2008) for 2004 in France.
e Data from (ter Laak *et al.,* 2010) for Germany, Switzerland and France.
f Data from (Besse *et al., 2012*) for 2008 in France.
g Data from (Vulliet and Cren-Olivé , 2011) for 2008 in France.

1.2. Pharmaceutical and personal care products (PPCPs)

Pharmaceuticals are a set of compounds, which have obtained increasing attention over the past decade. Pharmaceutical and Personal Care Products (PPCPs) are a set of chemical pollutants resulting from pharmaceutical and products for personal hygiene. They include a wide and diverse range of chemicals, including prescription drugs and medicines, perfumes, cosmetics, sunscreens, cleansers, shower gel, shampoo, deodorant and other. When these substances are freely discharged into the environment, they could cause some impact on aquatic and terrestrial organisms (Fent *et al.,* 2006; Jjemba, 2006), since they have been specifically designed to produce biological effects even at very low concentrations. This broad collection of substances includes any products consumed by individuals or domestic animals for any number of countless reasons pertinent to health, performance, cognitive and physical function, or appearance (Petrovic and Barcelo, 2007).
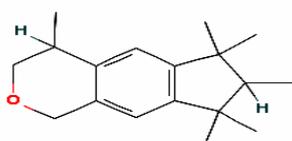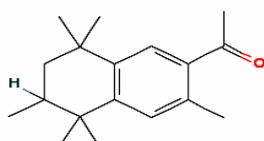
Galaxolide (HHCB)	Tonalide (AHTN)	Diclofenac (DCF)
Ibuprofen (IBP)	Naproxen (NPX)	Estrone (E1)
17β-estradiol (E2)	17α-ethinylestradiol	(EE2) Diazepam (DZP)
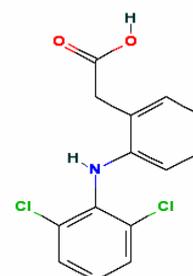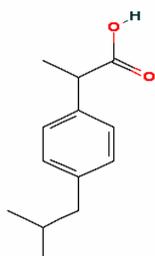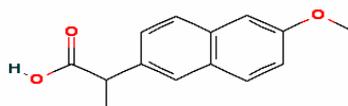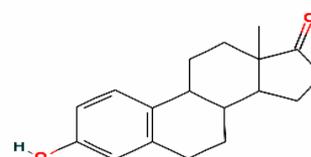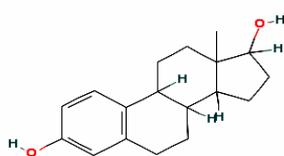
Carbamazepine (CBZ)   Sulfamethoxazole (SMX)   Iopromide (IPM)
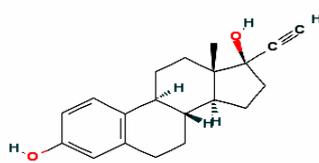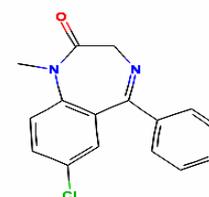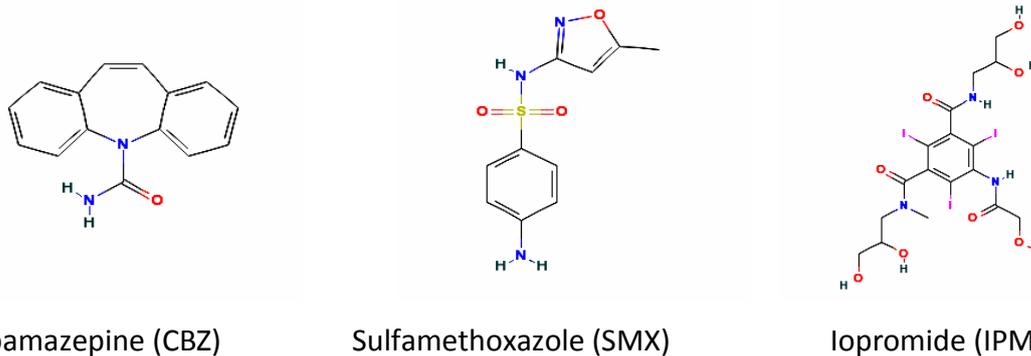
Figure1: Chemical Structure of selected PPCPs (http://pubchem.ncbi.nlm.nih.gov).

A few compound classes will be highlighted, either because the concentrations found in water are high, because of their (increasing) high volume usage or because of the persistence of these compounds.

1.2.1. Antibiotics

Antibiotics are widely used. Hospital wastewater effluents are one source of antibiotics, although wastewater effluents from tropical fish farm plants appeared to be also an important source of antibiotics (Kobayashi *et al.,* 2006). Some of these substances sometimes show low absorbance to sewage sludge (log Kow = 1 – 6) (Brown, 2004). Antibiotics such as sulfamethoxazole, trimethoprim, penicillin and caffeine were detected in hospital wastewater at high levels (0.3 – 35 μg/l). Only sulfamethoxazole, trimethoprim and ofloxacin were present in WWTP treated effluent in concentrations ranging from 0.11 to 0.47 μg/l. The substances trimethoprim and ofloxacin are part of the quinolone antibiotics (QAs) which have been widely used for the last 20 years in Europe and the United States (Nakata *et al.,* 2005). QAs consists of compounds such as pipemidic acid (PIP), ofloxacin (OFL), norfloxacin (NOR), ciprofloxacin (CIP), lomefloxacin (LOM), enrofloxacin (ENR), difloxacin (DIF), sarafloxacin (SAR), and tosufloxacin (TOS). Also antibiotics belonging to the quinolone group, including fluoroquinolones (FQs), are of particular environmental concern, because of the potential inhibition of DNA gyrase, a key enzyme in DNA replication (Bryan *et al.,* 1989). Ofloxacin, lomefloxacin, norfloxacin and ciprofloxacin are the QAs which are frequently found in WWTP effluents across Europe up to concentrations of 0.3 ug/L. Removal efficiencies of antibiotics in general were estimated between 20 to 70 percent in WWTPs, mainly due to the low (Kow) value of antibiotics (Log Kow ~ 1). Sulfamethoxazole,

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found in relatively high concentrations in hospital wastewater, displayed high persistence and is detected at concentrations up to 0.3 µg/L in WWTP effluents.(Schrap *et al.,* 2003).

1.2.2. Antineoplastic drugs

During the past years, the growing use of antineoplastic drugs in cancer therapy is an emerging issue in environmental research and it can be expected, that consumption will increase due to a developing health care system and a higher life expectancy. Cytostatics belong to the CMR (carcinogenic, mutagenic and reprotoxic) drugs. They usually enter the hospital effluents partially transformed or even unchanged via urine and faeces of patients under medical treatment. Therefore, they are assumed to be environmentally relevant compounds. As hospital effluents reach the municipal sewer network generally without any preliminary treatment, hospitals may represent an incontestable release source of anticancer agents. Besides, nearly 80% of cancers therapies are administered in the outpatient treatment ward, i.e. patients leave the hospital after drug application (Mahnik *et al.,* 2007). Subsequently, the drugs are also directly excreted into the municipal sewer network. Their quantification in hospital effluents may serve as a starting point to individualize the magnitude of potential pollution problems. Especially in Germany, investigators have been active in monitoring the fate of cytostatics in the environment after administration to patients. The concentrations of the antineoplastics cyclophosphamide and ifosfamide in the effluents of domestic WWTPs in Germany were determined to be between 6.2–8.5 ng/L and 6.5-9.3 ng/L respectively.64 In a WWTP of an oncologic hospital in Germany, much higher concentrations in the effluent were observed (0.006–1.9 µg/L and 0.02-4.5 µg/L respectively). No significant reduction during sewage treatment was observed. Treatment of oncologic wastewater in a membrane bioreactor resulted in concentrations below the limit of detection. Most anticancer drugs could be eliminated to a major extent (80%) by sewage treatment plants, either by biodegradation or adsorption.

1.2.3. Endocrine disrupters (EDCs)

Endocrine disrupters (also called hormonally active agents) are any type of chemical or mixture of chemicals that affect the endocrine system, and cause negative reproductive and developmental health effects for the human or animal and/or their offspring. The endocrine system is a complex network of organs, including the thyroid, pancreas, pituitary, ovaries,

testes, and adrenal glands, which secrete hormones into the bloodstream to target cell receptors in other organs or tissues, where the hormone has a specific effect. (Pontius, 2001; Symons *et al.,* 2000). In general, there are three major classes of endocrine disrupting compounds, which are estrogenic (compounds that mimic or block natural testosterone), androgenic (compounds that mimic or block natural testosterone), and thyroidal (compounds with direct or indirect impacts to the thyroid).

Estrogens

The most studied endocrine disruptors are those organic compounds, which mimic the hormone oestrogen. Oestrogenic steroids such as the synthetic steroid hormone 17a-ethynylestradiol (EE2) prescribed as oral contraceptive for birth control or oestrogen substitution therapies and the natural hormone 17s-estradiol (E2) and its main metabolite oestrone (E1) are among the most potent EDCs causing effects in aquatic organisms (Zuehlke *et al.,* 2005). Several studies have been performed on the determination of the oestrogen activity in WWTP effluents (Zuehlk *et al.,* 2005; Asmaa *et al.,* 2003; Johnson *et al.,* 2005; Desbrow *et al.,* 2011; Joss *et al.,* 2004) On several locations in Europe, (Belgium, Finland, France, Germany, Norway, Sweden, Switzerland and The Netherlands), the WWTP effluents and surface water have been studied for the presence of estrogens (STOWA, 2003).

Treatment processes included primary and chemical treatment only, but also more advanced treatment processes (e.g. ozone) have been studied. In all studies, significant levels of estrogens are detected in both WWTP influent- and effluent water, ranging from 2 up to 51 ng/L and from 0.5 to 3 ng/L, respectively. The highest estrogen values were detected in the effluent of the WWTPs which only used primary treatment (35 ng/L E1, 13 ng/L E2 and 0.05-1,6 ng/L EE2). For WWTPs equipped with a secondary treatment, the concentration of E1 and E2 in the effluent was between 0.7–5.7 ng/L and 0.8-3.0 ng/L respectively. The removal efficiency of E1 and EE2 clearly depends on the redox conditions of the purification process. This is partially due to the reduction during this process of E1 into E2. A biological degradation of more than 90% of the E1, E2, and EE2 load can be expected from conventional activated sludge plants and membrane bioreactors. The removal efficiency of estrogens is improved when sludge retention times increases (Joss *et al.,* 2004). This can be ascribed to the relatively moderate (log Kow) values of estrogens of 3-4 and a

very low vapour pressure (Henry constant). The concentration of estrogens in WWTP effluents is found to be proportional to the population numbers of the city associated with the specific WWTP. For example, the stretch of the River Elbe between Dresden and Magdeburg has some big population centres and associated endocrine disrupting effects in the resident fish in some regions have now been detected. In these areas, the addition of tertiary treatments, known to reduce micro-organic pollutants in drinking water purification, such as ultra filtration, ozonation, UV treatment, activated charcoal etc. may need to be considered for the removal of estrogens.

1.2.4. General pharmaceuticals

Anti-inflammatories and analgesics, lipid regulators and s-blockers are the major groups detected in WWTP effluents across Europe and among them are acetaminophen, ketoprofen, ibuprofen, diclofenac, mevastatin, atenolol, propranolol, sulfamethoxazole, bezafibrate and trimetroprim as the most abundant, with concentrations at levels (Petrovic et al., 2006; Lishman *et al.,* 2006). The highest concentrations were detected for acetaminophen (paracetamol) and for trimethoprim, with average concentrations in WWTP effluent of 2.1 μg/L and 0.29 μg/L respectively, (Meritxell *et al.,* 2006). Other compounds frequently detected in WWTP samples were carbamazepine and ranitidine, with average concentrations of 400 ng/L for carbamazepine and 135 ng/L for ranitidine in effluent (Ternes, 1998). Different removal behaviour was observed for the investigated compounds. Some compounds as the antiepileptic drug carbamazepine were not removed at all in any of the sampled treatment facilities and effluent concentrations in the range of influent concentrations were measured. Other compounds as bisphenol- A, the analgesic ibuprofen or the lipid regulator bezafibrate were nearly completely removed. The drugs detected in the environment were predominantly applied in human medicine. Due to their widespread presence in the aquatic environment many of these drugs have to be classified as relevant environmental chemicals (Vogelsang *et al.,* 2006).

1.2. 5. Musk fragrances

Synthetic musks are a group of chemicals possessing a chemical structure that is not readily biodegradable they are capable of being bio- concentrated in aquatic organisms (Clara *et al.,* 2005 a; Balk and Ford, 1999; Carlson *et al.,* 200). The most frequently used synthetic musks

are Musk ketone: 1-tert.-Butyl-3,5-dimethyl-2,6-dinitro-4-acetylbenzene (MK); Musk moskene: 4,6-Dinitro-1,1,3,3,5-pentamethylindane (MM); Musk ambrette: 2,6-Dinitro- 3-methoxy-4-tert.-butyltoluene (MA); Musk xylene: 1-tert.-Butyl-3,5-dimethyl-2,4,6-trinitrobe-nzene (MX) and Musk tibeten: 1-tert.-Butyl-2,6-dinitro-2,4,5-trimethylbenzene (MT).

The Log Kow values of these compounds and their metabolites vary from 4.3 to 6.3 and from 4.8 to 5.1 respectively. These synthetic compounds are used as more affordable substitutes for the expensive natural musks (e.g., muscone, civetone, and ambrettolide) present in many perfumes. Based on this (Log Kow) most of these musks will be more or less efficiently removed by a WWTP treatment. Many manufacturers voluntary are replacing the older and more toxic substances for newer, such as tonalide (AHTN) and galaxolide (HHCB).

There are four synthetic musk fragrances accounting for 95% of the total amount used. These are the nitro-musks (musk xylene, used in detergents and soaps, and musk ketone, used in cosmetics) and two polycyclic musks HHCB and AHTN. Synthetic musks enter city sewage systems (presumably from bathing, laundry detergents, and other washing activities), and then the aquatic ecosystem, where they may potentially bio-concentrate and bio-magnify in the tissues of aquatic organisms. Fragrances are reported in several studies and they are identified in effluents and surface water (Bitsch *et al.,* 2002; Jossa *et al.,* 2005). Concentrations up to 0, 73 mg/l are found in effluents of domestic WWTP (Jossa *et al.,* 2005). Two nitro musks (musk xylene, musk ketone), a major metabolite of musk xylene and the polycyclic musk fragrance tonalide (AHTN) are suspected of having estrogenic activity (Bitsch *et al.,* 2002). It has been established that the partial removal observed for the two fragrances AHTN and galaxolide (HHCB) during wastewater treatment is mainly due to sorption (log KOW > − 4.9) onto sludge and not to biological transformation. Due to the incomplete removal of fragrances in conventional WWTP, the ozonation has been tested as a possible tool for the enhanced removal of fragrances. By applying 10 −15 mg/l of ozone (contact time: 18 min), most of the musk fragrances were no longer detected (Bitsch *et al.,* 2002).

1.2.6. Sunscreen Agents (SSAs)

Sunscreen agents (SSAs) are more and more widely used for protection against harmful UV radiation. The concentration of these sunscreen agents in water is limited (0.004 µg/L) and considerable concentrations are found in aquatic organisms (21 µg/kg) indicating that SSAs are able to bio-concentrate (Nagtegaal *et al.,* 1997). The fact that SSAs (e.g., oxybenzone (2-hydroxy-4-methoxybenzophenone) and 2-ethylhexyl-4-methoxycinnamate) can be detected in human breast milk shows the potential for (dermal) absorption and bioconcentration in aquatic species (Hany and Nagel, 1995). No data have been published on more recently used SSAs such as avobenzene.

1.2. 7. Diagnostic contrast media

There are two basic types of contrast agents used; one type is based on barium sulfate, the other type on iodine. Triiodinated benzene derivatives are widely used as X-ray contrast agents. The preferential uptake of triiodinated compounds in specific organs enhances the contrast between those organs and the surrounding tissues and enables the visualization of organ details which otherwise could not be investigated. The compounds may be bound either as an organic (non-ionic) compound or as an ionic compound. Ionic agents were developed first and are still in widespread use depending on the examination they are required for. Most commonly used X-ray contrast media are: Diatrizoate (Hypaque 50), Metrizoate (Isopaque Coronar 370), Ioxaglate (Hexabrix), Iopamidol (Isovue 370), Iohexol (Omnipaque 350), Iopromide, Iodixanol (Visipaque 320) (Putschew *et al .,* 2000). These contrast media are applied by intravenous injection and are rapidly eliminated via urine or faeces. Due to the high hydrophicity of the substituted benzene derivatives (Log Kow = -2) they pass wastewater treatment plants without any cleavage and thus, are found in rivers, lakes and even raw drinking water (Putschew *et al.,* 2000; Daughton and ternes, 1999). The contrast agent diatrizoate occurs with concentrations up to 5.2 µg/L as is iopromide found in concentrations up to 5.7 µg/L in effluents of WWTPs, (Ternes *et al.,* 2003). These are the most abundant and most used iodated contrast media (ICMs). In specific effluents of WWTPs near hospitals, the concentrations of ICMs can be much higher (up to 1200 µg/L), (Schrap *et al.,* 2002).

Secondary treatment and introduction of oxidation steps only enhance the removal efficiency of these iodated agents in a limited way. Even with a 15 mg/L ozone dose, the ionic diatrizoate only exhibited removal efficiencies not higher than 14%, while the non-ionic ICM (diatrizoate, iopamidol, iopromide and iomeprol) were removed to a degree of higher than 80%. Advanced oxidation processes (e.g. O_3/H_2O_2), which were nonoptimized for wastewater treatment, did not lead significantly to a higher removal efficiency for the ICM than ozone alone. It is interesting to note the high variation of the influent concentrations for iopromide: the fact that the influent load in a WWTP serving 120,000 population equivalents can vary by more than a factor of seven from one 24 h composite sample to the next suggests that most of this compound is emitted irregularly by a small number of point sources (Jossa *et al.,* 2005). The metabolites of these contrast media have not been identified yet. The evaluation of the ecotoxicity of triiodinated contrast agents must include the transformation products. No environmental risk has to be expected from the triiodinated contrast media itself, Steger-Hartmann et al., 1999), but the metabolites may have an ecotoxicological impact. Most likely, the transformation products carry free amino groups, which might be mutagenical, thus, identification of the transformation products is very important (Kalsch, 1999).

1.3. Sources, pathways and fates the PPCPs

After a chemical is created, the route that it takes between initial observation and final observations is referred to as a pathway. Common pathways include manufacture to initial use, initial used to disposal and initial use to release to the environment. The result of interactions between a chemical compound and its environment over a series of events and procedures is known as its fate. Even though a number of research publications have been focused on the occurrence, fate, and effects of pharmaceuticals in the environment, we have data on the occurrence of only 10% of the registered active compounds, and very little information on their effects in the environment. There is even less information regarding the occurrence and fate of the transformation/degradation products (active or not) of pharmaceuticals. Both the qualitative and the quantitative analysis of pharmaceuticals in the environmental matrices are definitely a starting point for the establishment of new regulations for the environmental risk assessment of pharmaceutical products. Discharge of PPCPs can occur from domestic wastewater, hospital wastewaters or industrial discharges.

Hospitals are important sources of these compounds: a great variety of micro contaminants result from diagnostic, laboratory and research activities on one side and medicine excretion by patients on the other. They include active principles of drugs and their metabolites, chemicals, heavy metals, disinfectants and sterilizants, specific detergents for endoscopes and other instruments, radioactive markers and iodinated contrast media. But hospitals are not the only source: residues of pharmaceuticals can be found in all wastewater treatment plant (WWTP) effluents, due to their inefficient removal by conventional systems (Kummerer, 2001; Petrovic *et al.,* 2003; Carballa *et al.,* 2004; Onesios *et al.,* 2009).

Despite their specific nature, quite often hospital effluents are considered to be of the same pollutant load as urban wastewaters (UWWs) and are discharged into public sewer networks, collected to a WWTP and co-treated with UWWs. Before entering into the municipal sewer, chlorination is sometimes required for the whole hospital wastewater flow rate, sometimes only for the effluent from infectious disease wards (Emmanuel *et al.,* 2004). PPCPs eventually enter wastewater treatment plants (WWTP). During wastewater treatment, a distribution occurs between the dissolved and solid phases. Influent suspended solids are largely removed through primary clarification. The separation is relevant for the most lipophilic compounds. As a result, non-degraded PPCPs will be discharged into the environment not only through the final effluent of the plant, but also with biosolids. (Kinney *et al.*, 2006) showed that organic wastewater contaminants could be detected in the target biosolids with high occur frequency and high concentration, which suggests that biosolids can be an important source of organic wastewater contaminants to terrestrial environment. (Xia *et al.,* 2005) indicated that the PPCPs that enter wastewater treatment plants can undergo partial or complete transformation and by-products can be discharged to the environment in the final effluent or through biosolids being applied to land. One of the main sources of emerging contaminants is untreated urban wastewater and effluents from wastewater treatment plants. Most current wastewater treatment plants are not designed to treat these compounds. (Heberer *et al.,* 2002) identified diclofenac as one of the most important pharmaceuticals in the water cycle, with low mg/L concentrations in both row and treated wastewater (3.0 and 2.5 mg/L at the influent and effluent, respectively). Atenolol, metoprolol, and propranolol have been frequently identified in wastewaters, where atenolol was detected in the highest concentrations, in some cases ranging up to 1 mg/L. As a result

of the incomplete removal during conventional wastewater treatment, these compounds were also found in surface waters in the ng/L to low mg/L range (Ternes *et al.,* 1998).

Antibiotics are destined to treat diseases and infection caused by bacteria. They are among the most frequently prescribed drugs for humans and animals in modern medicine. Beta-lactams, macrolides, sulfonamides, fluoroquinolones, and tetracyclines are the most important antibiotic groups used in both human and veterinary medicine. High global consumption of up to 200,000 tons per year (Kummerer, 2003) high percentage of antibiotics that may be excreted without undergoing metabolism (up to 90%) result in their widespread presence in the environment (Huang *et al.,* 2001). Unmetabolized pharmaceutically active forms of antibiotics concentrated in raw sludge may promote the development of bacterial resistance. Bacteria in raw sludge are more resistant than bacteria elsewhere (Jones et al., 2004). Many active antibiotic substances were found in raw sewage matrices, including both aqueous and solid phase. Sulfonamides, fluoroquinolone, and macrolide antibiotics show the highest persistence and are frequently detected in wastewater and surface waters (Huang *et al.,* 2001). Sulfamethoxazole is one of the most detected sulfonamides (Brown *et al.,* 2006; Yang *et al.,* 2005) that was reported with various concentrations and up to ca. 8mg/L (in raw influent in China) (Peng *et al.,* 2006). Sulfamethoxazole is often administrated in combination with trimethoprim, and commonly analyzed together (Gobel *et al.,* 2005). The class of tetracyclines, widely used broadspectrum antibiotics, with chlortetracycline, oxytetracycline, and tetracycline as mostly used, was detected in raw and treated sewage in many studies in the ng/L (Kim *et al.,* 2005) to mg/L concentrations (Yang *et al.,* 2003). Tetracyclines and fluoroquinolones form stable complexes with particulates and metal cations, showing the capacity to be more abundant in the sewage sludge (Alexy *et al.,* 2004; Daughton *et al.,* 1999). Some of the most prescribed antibiotics—macrolides clarithromycin, azithromycin, roxithromycin, and dehydro-erythromycin were found in various environmental matrices in a variety of concentrations from very low ng/L to few mg/L (Gobel *et al.,* 2005; Karthikeyan *et al.,* 2006).

While antiepileptic carbamazepine is one of the most studied and detected pharmaceuticals in the environment, there is not much information on the occurrence and fate of other of psychoactive drugs in WWTPs. Carbamazepine is one of the most widely prescribed and very

important drug for the treatment of epilepsy, trigeminal neuralgia, and some psychiatric diseases (e.g., bipolar affective disorders (Fertig *et al.,* 2008; Yoshimura *et al.,* 1998). In humans, following oral administration, it is metabolized to pharmacologically active carbamazepine-10, 11-epoxide, which is further hydrolyzed to inactive carbamazepine-10, 11-trans-dihydrodiol, and conjugated products which are finally excreted in urine. Carbamazepine is almost completely transformed by metabolism with less than 5% of a dose excreted unchanged (Shorvon *et al.,* 2004). In fact, carbamazepine and its metabolites have been detected in both wastewaters and biosolids (Miao *et al.,* 2005). Carbamazepine is heavily or not degraded during wastewater treatment and many studies have found it ubiquitous in various environment matrices (groundwater, river, soil) (Metcalfe *et al.,* 2003; Radjenovic *et al.,* 2007; Clara *et al.,* 2005; Zhang *et al.,* 2008). The concentrations of carbamazepine vary from one plant to another, and they are usually around hundreds ng/L, and in some cases also few mg/L (Ternes *et al.,* 2005; Ternes, 1998). As a result, a high portion of emerging contaminants and their metabolites can pass through the treatment process and enter the aquatic environment via wastewater effluents without any elimination. (Figure 2)
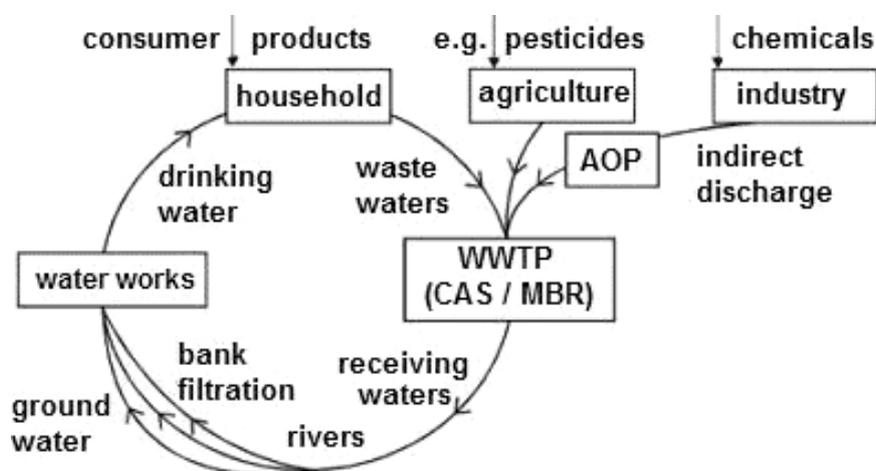
Figure 2: Pathways of emerging contaminants (Buttiglieri *et al.,* 2007)

1.4. Chemical and physical properties of PPCPs

When examining the fate, pathways, and partitioning of emerging contaminants, it is important to consider the physical and chemical properties of each compound (table 3). By studying the physical and chemical properties of chemical compounds it is possible to predict their fate in some situations. One commonly used class of physical properties is

partitioning coefficients. Partitioning refers to the tendency of a chemical to concentrate in one phase of a two-phase mixture at equilibrium. The mixture can be two liquid phases, a liquid and a solid phase, a liquid and a gas phase, or a combination thereof. A partitioning coefficient is the dimensionless ratio of concentrations present in the two different phases of a two-phase mixture. The octanol-water partitioning coefficient is a measure of the partitioning between octanol and water, which describes the hydrophobicity of a compound and is inversely related to the solubility of a compound in water. Compounds with a high Kow have been shown to preferentially adsorb to soil and sediment particles in water (Karickhoff *et al.,* 1979).

Similarly, a sludge adsorption coefficient or Kd, is a ratio of the amount of compound adsorbed to sludge compared to the amount present in aqueous solution under the specific conditions the measurement was taken. In water and wastewater treatment, the sludge adsorption coefficient is commonly used to predict the extent to which a compound can be removed by physical adsorption to sludge particles in a primary or secondary clarification unit. A commonly use chemical property is the acid dissociation constant of Ka. It is a measure of the strength of an acid in solution and is the concentration ratio of ionized to un-ionized species of a compound at equilibrium. The Ka of a compound enables the concentration of ionized or un-ionized versions of a chemical to be calculated for a given pH. Due to the large range in magnitudes of Kas, the logarithmic constant (pKa) is commonly used.

Table 3: Physical and Chemical Properties of Selected Emerging Contaminants

Compound or class	Molecular Weight (g/mol)	Acidity constant (Pka)	Octanol- water Coeffcient (log Kow)	Water solubility (mg/L) at 25C°
Beta Blockers				
Sotalol	272,4	9.5	0.24	137
Atenolol	266.3	9.2	0.16-0.46	13,3
Metoprolol	267.4	9.7	1.69-1.88	4,78
Propranolol	259.3	9.45	3.48-3.03	70
Disinfectants				
Triclosan	289.5	7.9	4.8	2- 4.9
Triclocarban	315.6	12.7	4.3	0.6-1.5
Antidepressants				
Venlafaxine	277.41	9.24	0.43	-
O-desmeth ylvenlafaxine	263.38	9.74	0.74	-
Citalopram	324.4	9.5	3.74	-
desmethylcitalopram	310.37	10.5	-	-
Musks				
Musk ketone	294.3	-	4.3	1.9
Musk xylene	297.3	-	4.9	0.49
Galaxolide	258.4	-	5.9	-
Tonalide	258.4	-	5.7	-
Antibiotics				
Erythromycin	733	8.9	3.06	2000
Pharmaceuticals				
Naproxen	230.3	4.2	3.18	5.11
Carbamazepine		13.9	2.25	-
Ibuprofen	206.3	4.9	3.5	-
Estrogens				
Estrone	270.4	10.7	3.13	800
Bisphenol A	228.3	10.3	2.2-3.4	120
Perfluorinated Compounds				
Perfluorooctanoate	414.0	3.8	6.3	-
PBDEs	-	4-10.9	-	-

1.5. Problematic statement and toxicity of hospital wastewater to the environment

In 2011, the World Health Organization published a report entitled "Pharmaceuticals in drinking-water" which reviews the risks to human health associated with exposure to trace concentrations of pharmaceuticals in drinking-water. But, the effect and hazard of emerging contaminants to public health and environment are poorly understood. Human and veterinary applications are the main sources of PPCPs in the environment that are introduced primarily through excretion and the subsequent transport in sewage, whereas direct disposal of unwanted or expired drugs in the sewage is believed to be of minor importance (Heberer, 2002a). These chemicals are designed to have a specific mode of

action, and they have varying persistence in the body. These features among others suggest that it is important to evaluate the effect of pharmaceuticals on aquatic flora and fauna. PPCPs in the environment lately have been acknowledged to constitute a major health risk for humans and members of terrestrial and aquatic ecosystems (Bendz *et al.,* 2005).

Ecotoxicity of emerging contaminants can be divided in to two aspects: acute and chronic. The present research indicates that LC50 or EC50 concentrations for PPCPs such as fluoxetine and diazepam are approximately 100 times greater than commonly observed environmental concentrations. There is a general lack of chronic toxicity data on pharmaceuticals, in particular in fish. Many pharmaceuticals need more investigation to determine potential long-term ecotoxicological effects, particularly with respect to potential disturbances in hormonal homeostasis (endocrine disruption), immunological status, or gene activation and silencing during long-term exposure (Fent *et al.*, 2006). Many PPCPs do not exhibit an acute aquatic toxicity but have a significant cumulative effect on the metabolism of nontarget organisms (Halling-Sorensen *et al.,* 1998) and the ecosystem as a whole (Daughton and Ternes, 1999). Many endocrine disruptors induce serious effects in low concentrations (Heberer, 2002a; Halling-Sorensen *et al.,* 1998; Jorgensen and Halling-Sorensen, 1998) but also individual PhCs occurring in low concentrations may exhibit synergistic and cumulative effects. In addition, the development of antibiotic resistance may be stimulated in bacteria from exposure to low concentrations (Jorgensen and Halling-Sorensen, 1998). (Baquero *et al.,* 2008; Kummerer, 2004) investigated that HWW is a source for undesirable constituents, such as (multi-)antibiotic-resistant bacteria. According to the Centers for Disease Control and Prevention, about 2 million people in hospitals get infections each year, which cause 90,000 deaths. Of these, more than 70 percent of the bacteria that causes these infections are resistant to at least one common antibiotic that is typically used to treat them.

(Emmanuel, 2001) was confirmed that the hospital effluents have generally a very weak microbiological load resulting from the regular use of disinfectants. These bactericides can have a negative influence on the biological processes of the WWTP. Even by considering that these effluents are diluted after their discharge towards the municipal WWTP, it remains evident that it is not necessary to neglect the possibility that certain substances present in

the WWTP effluents can generate by cumulative effect a biological imbalance in aquatic ecosystem. To protect the natural environment against the phenomenon of excess load in the processes of the WWTP, it seems important to consider upstream treatments of hospital wastewater before their discharge in the municipal sewage system. As a result, it has been suggested in some studies that pre-treatment of HWW prior to discharge into the sewers provides a reasonable solution (Gautam *et al.,* 2007; Lenz *et al.,* 2007b; Pauwels and Verstraete, 2006).

2. Removal of Pharmaceutics Compounds by Treatment Technologies

2.1. Removal mechanism

2.1.1. Volatilization

Volatilization was an important removal mechanism for the low-molecular weight compounds in the basins; between 30 and 70% of the chlorinated benzenes and 1- and 2-carbon halogenated organic compounds were removed in this way (Yu *et al.,* 2006). The process converts a chemical substance from a liquid or solid state to a gaseous or vapour state. The fraction of compound volatilized in the aeration tank depends on the flow of air getting in contact with wastewater (Qair), type of aeration and Henry coefficient (H), as shown in Eq. 1 (Suarez *et al.,* 2008).

$$\emptyset = \frac{C_{soluble}.\,H.\,Q_{air}}{C_{soluble} + C_{soluble}.\,H.\,Q_{air} + C_{soluble}.\,K_d.\,SS} = \frac{H.\,Q_{air}}{1 + H.Q_{air} + K_d.\,SS} \qquad (Eq.1)$$

Considering about the typical aeration rate and the Henry coefficient of selected PPCPs, the removal for ADBI due to volatilization is quite significant, but is negligible for pharmaceuticals, estrogens, AHTN, and HHCB.

2.1.2. Sorption

Solid-Water distribution coefficient is commonly used to determine the fraction of PPCPs sorbed sludge solute is introduced into any two phase system, such as soild/water, distribution coefficient (Kd, L/kg) is calculated as the ratio of the concentration of the PPCPs in one phase to the concentration of the PPCPs in the other phase under equilibrium conditions (Eq.2) (Ternes *et al.,* 2004)

$$K_d = \frac{C_{sorbed}}{SS.\ C_{soluble}} \qquad \text{(Eq.2)}$$

Where C_{sorbed} is the sorbed PPCP concentration onto sludge (µg/L), $C_{soluble}$ the dissolved concentrate on of the compound (µg/L) and SS the suspended solids concentration (kg/L). There are two main sorption mechanisms influenced by distribution coefficient:

Absorption: It is a process refers to the hydrophobic interactions of the aliphatic and aromatic groups of a compound with the lipophilic cell membrane of the microorganisms and the lipid fractions of the sludge. The lipophilicity of substances is related to the octanol-water partition coefficient (Kow). Polycyclic musk fragrances (galaxolide, tonalide, and Celestolide) are the most common lipophilic compounds among PPCPs.

Adsorption: it is the process of accumulating substances that are in solution on a suitable interface. Electrostatic interactions of positively charged groups of chemicals with the negatively charged surfaces of the microorganisms force ions and molecules to bind on the surface or another molecule. Therefore, the tendency of a substance to be ionized or dissociated will influence the efficiency of adsorption. The degree of ionization or dissociation could be characterized by dissociation constant (Ka). In general, cationic species of PPCPs will be more intend to be adsorbed due to Van der Waals interactions, and negatively charged molecules will not be adsorbed. The sorption coefficient (Kd), pseudo first-order degradation constant (kbiol), and octanol-water partition coefficient (Kow) values of emerging contaminants frequently found in wastewater treatment plants are given in Table (3). According the statement above, both octanol-water partition coefficient (Kow) and dissociation constant (Ka) could affect the sorption intendancy of PPCPs. Comparing the properties of selected PPCPs, several phenomena could be illustrated as following: (1) Polycyclic musk fragrances (HHCB, AHTN, ADBI) have high log Kd values (33.3-3.9), which consist with their low solubility in water. The strong lipophilic character could be indicated by high log Kow values (4.6-6.6). (2) Compared with musk fragrances, the selected hormones in Table 3 have both lower log Kow values (2.8-4.2) and sorption coefficients (log Kd of 2.3-2.6). Therefore, they have weaker interaction with sludge. (3) The sorption capacity of the

antibiotic trimethoprim (TMP) is similar to that of the previously cited hormones, although in this case the interaction with sludge is mainly driven by adsorption, since this compound is not lipophilic, but at circumneutral pH the dicationic species of TMP account for approximately 50% of the total TMP concentration (Suarez *et al.,* 2008).

(Park *et al.,* 2009), but no clear correlation has yet been found due to the great variability of compounds and their behaviour. Thus, graphs plotting the percentage removal rate vs. molecular weight or vs. log Kow yield clouds of data, showing a wide variability in the behaviour of the substances considered. For instance, some pharmaceuticals contain planar aromatic structures, which favour intercalation, for example into the layers of some clay minerals. Therefore, the sorption of such compounds depends not only on the log Kow, which is the lipophilicity of the sorbed molecule, but is also governed by pH, redox potential, stereochemical structure and the chemical nature of both the sorbent and the sorbed molecule (Kummerer, 2009). Experimental data on PPCPs concentration in sludge are very rare. The possible reason of that could be the difficulty of analysis these compounds precisely in sludge. To overcome this problem, distribution coefficient (Kd) seems to be a useful tool to predict distribution between solid and water phases. However, since Kd is influenced wastewater farm by several parameters, such as the characteristics of the solid phase (organic carbon content, particle size), and experimental conditions in which sorption is studied (sorbate and sorbent concentrations, pH, salinity, ions content), an accurate determination of this coefficient under several environmental conditions is needed (Carballa *et al.,* 2007).

2.1.3. Biological degradation

During biological degradation in wastewater treatment plants, pharmaceutical contaminants may be transformed into either more hydrophobic compounds, which could be adsorbed onto the solid surface of the activated sludge, or more hydrophilic compounds, which remain in the liquid phase and will eventually be discharged into aquatic environment. Even there are various groups of microorganisms in the activated sludge, it is unlikely that pharmaceuticals present as micro contaminants in wastewater can be effectively removed by biodegradation alone for three reasons. First, compared with other pollutants in wastewater, pharmaceutical contaminants have relatively low concentration, which may be

insufficient to induce enzymes that are capable of degrading pharmaceuticals. Second, since many of the pharmaceutical contaminants are bioactive and this characteristic can inhibit metabolism of microorganisms. As result, it is impossible that pharmaceutical contaminants can be used as favourable carbon or energy sources by microorganisms.

Third, the nature of each compounds and the operating condition of wastewater treatment plant will influence the performance of biodegradation.

(Joss et al., 2006) conveyed a research using activated sludge to investigate the biodegradation of 25 pharmaceuticals, hormones and fragrances, including antibiotics, antiphlogistic, contrast agent , lipid regulator, and nootoropics, in batch experiments at typical concentration levels in a municipal wastewater treatment. He indicated that only a few compounds, which were ibuprofen, paracetamol, 17β-estradiol, and estrone, could be degraded by more than 90%, while half of the target compounds were removed by less than 50%. Joss also determined pseudo first-order degradation kinetics (kbiol) for all target compounds down to ng/L levels.
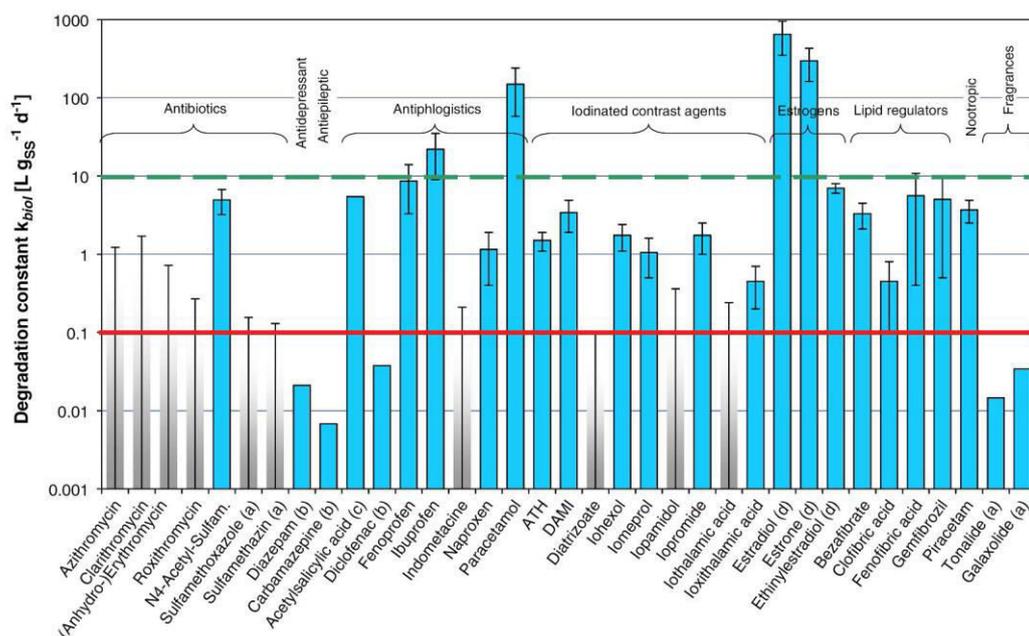
Figure 3: Kinetic degradation constant of 35 pharmaceuticals, hormones, and personal care products. (Joss *et al.,* 2006)

Figure 3 shows the degradation constant (kbiol) of 35 PPCPs observed in Joss's study. According to the degradation constant (kbiol) of target compounds, the contaminants could

be divided into three groups: (1) highly degradable, with $K_{biol}>10$ $Lg^{-1}SSday_{-1}$, such as paracetamol; (2) hardly degradable, with $K_{biol}<0.1$ $Lg^{-1}SSday^{-1}$, such as carbamazepine; (3) moderate degradable, with $0.1 < K_{biol}<10$ $Lg^{-1}SS.day^{-1}$, such as iohexol. It also seems that anoxic conditions favour the biodegradation of micropollutants as they promote biogeochemical reactions. (Park et al., 2009) suggest the necessity for further investigations into the removal mechanisms of micropollutants via biodegradation under anoxic conditions. For example, the biological transformation of amide and urea functional groups (attached to atenolol, carbamazepine, dilantin) via mediated hydrolysis reactions has been documented (Matamoros *et al.,* 2008).

However, aerobic transformations are generally faster than anaerobic ones for low-chlorinated compounds, while for polyhalogenated compounds aerobic degradation rates are slower. Furthermore, highly chlorinated substances, like diclofenac, are known to be biodegraded via a microbe-mediated reduction (Mohn and Tiedje, 1992; Schwarzenbach *et al.,* 2003; Matamoros *et al.,* 2007b; Matamoros *et al.,* 2008). These compounds characterized by their low water solubility, become more soluble, and therefore more bio available, after some initial reductive dechlorination steps. Under anaerobic conditions, however, microbial degradation takes time and a subsequent aerobic degradation step is necessary for breaking down the remaining carbon skeleton. According to (Jones *et al.,* 2005a), long and highly branched side chains render a compound more persistent, whereas unsaturated aliphatic compounds are more biodegradable than saturated or aromatic ones featuring complex ring structures and sulphate or halogen groups.

The Byrns model (Byrns, 2001) concerning xenobiotics degradation shows that at low SRTs, most of the compounds are removed through sludge discharge. As the SRT increases, the proportion of sludge wasted from system decreases and higher amount of less polar micropollutants remain in the system for further degradation.

(Kloepfer *et al.,* 2004a) was confirmed that two MBRs operated at high SRT of 26 d showed removal efficacy of 43% for benzothiazoles. By varying the SRT in MBRs, (Lesjean *et al.,* 2005) noticed that the removal of pharmaceuticals residues increased with a high sludge age of 26 d and inversely decreased at lower SRT of 8 d. (Ternes *et al.,* 2004) demonstrated that

SRT values between 5 and 15 d are required for biological transformation of some pharmaceuticals, i.e., benzafibrate, sulfamethoxazole, ibuprofen, and acetylsalicylic acid.

(Urase *et a.,* 2005) identified the pH value as critical parameter affecting the removal of micropollutants during the MBR treatment, pH value varied from neutral to acidic as nitrification became significant in the MBR At pH lower than 6, high removal rate (up to 90%) was observed for ibuprofen. Ketoprofen was removed from MBR up to 70% when the pH dropped down below 5. (Kim *et al.,* 2005) was found that at pH 6–7 tetracycline are not charged and therefore, adsorption sludge becomes an important removal mechanism.

Studies investigating the removal of pharmaceutics compounds from wastewater have been conducted. The various treatment technologies have been developed so far for treatment of hospital wastewater. Some of these treatment technologies include Conventional Activated Sludge (CAS), Membrane Bio-Reactor (MBR), Fluidized Bed Reactor (FBR), and membrane bioreactor coupled with granular and powder activated carbon. A brief description of each technology and their comparative analysis is given below.

2.2. Treatment technologies for hospital wastewaters

2.2.1. Conventional Activated Sludge Process (CAS)

CAS is very old technology. The basic mechanism of activated sludge process treatment is to use microorganisms to feed on organic contaminants in wastewater, producing a high quality effluent. The main principle behind all activated sludge processes is that as microorganisms grow, they form particles that clump together. These particles (floc) are allowed to settle to the bottom of the tank, leaving a relatively clear liquid free of organic materials and suspended solids (Metcalf and Eddy, 2003). Screened wastewater is mixed with varying amounts of recycled liquid containing a high proportion of organisms taken from a secondary clarifying tank, and it becomes a product called mixed liquor. This mixture is stirred and injected with large quantities of air, to provide oxygen and keep solids in suspension. After a period of time, mixed liquor flows to a clarifier where it is allowed to

settle. A portion of the bacteria is removed as it settles, and the partially cleaned water flows on for further treatment. The resulting settled solids and the activated sludge are returned to the first tank as the process continues.

The pollutants can be found in different forms, e.g. particulate, bound to colloids and dissolved. Depending on the type of pre-treatment a certain amount of pollutants in a certain form will enter the bioreactor. The treatment was originally designed for removal of COD and suspended solids. Due to problems with eutrophication of surface waters further treatment was required which included removal of nutrients like nitrogen and phosphorus. Next to the nutrients there is a long list of micro pollutants that will have to be removed from the wastewater. Special attention will have to be paid to the removal of medicine residues as well as endocrine disruptors, pesticides and heavy metals. A schematic overview of a typical conventional activated sludge process is presented in (Figure 4).
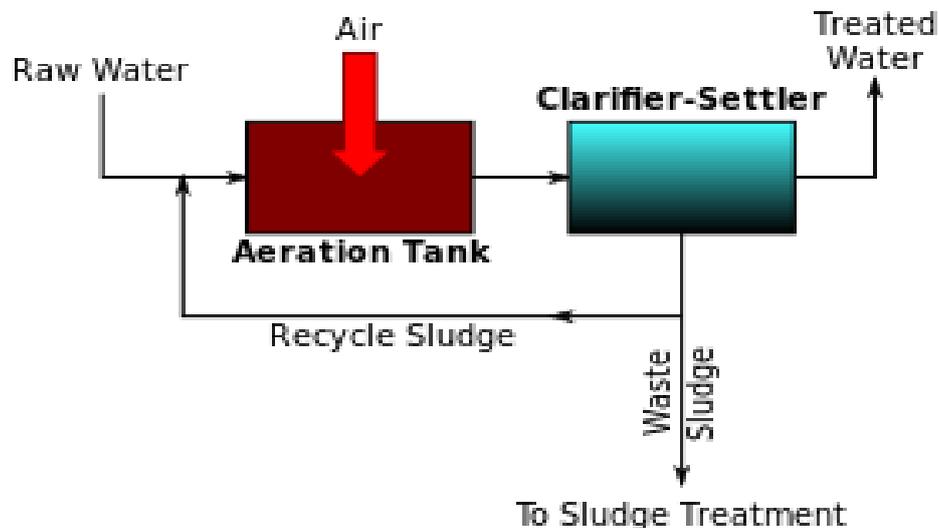
Figure 4: Schematic overview of a typical conventional activated sludge process

2.2.1.1. Removal of pharmaceutics compounds by Conventional Activated Sludge (CAS)

Municipal wastewater treatment plants were basically designed to remove pathogens and organic and inorganic suspended and flocculated matter. Even though the new treatment technologies have been developed to deal with health and environmental concerns associated with findings of nowadays research, the progress was not as enhanced as the one

of the analytical detection capabilities and the pharmaceutical residues remain in the output of WWTPs. (Jelic´ *et al.,* 2012).

Ternes (1998) monitored 32 pharmaceutical drugs and 5 metabolites in municipal WWTP influent and effluent, and in the receiving surface waters. The WWTP monitored in Frankfurt/Main Germany had three principle treatment steps: preliminary clarification, secondary aeration with Fe (II) chloride for phosphate elimination, and clarification. Generally, the WWTP removed greater than 60% of the pharmaceuticals. Only carbamazepine, clofibric acid, phenazone, and dimethylaminophenazone had lower than average removal rates. The study did not differentiate whether removal occurred by sorption or biodegradation. Over 80% of the pharmaceuticals were detectable in at least one effluent sample. Twenty pharmaceuticals and 4 metabolites were detected in the receiving surface water. Ternes found mainly the acidic drugs ubiquitously in surface waters in the nanogram-per-liter range. Acidic drugs include lipid regulators (bezafibrate, gemfibrozil), the antiphlogistics (diclofenac, ibuprofen, indomethacine, naproxen, phenazone, and the metabolites clofibric acid, fenofibric acid and salicylic acid) as well as neutral or weakly basic drugs such as the beta blockers (metoprolol, propranolol), and the antiepileptic drug carbamazepine. Flocculation with iron (III) chloride showed no significant removal of any of the five pharmaceuticals tested (Ternes et al., 2002).

➢ Diclofenac (4%)
➢ Clofibric acid (13%)
➢ Bezafibrate (11%)
➢ Carbamazepine (13%)
➢ Primidone (10%)

(Khan and Ongerth, 2004) developed a conceptual model for determining which pharmaceutical compounds would most likely be found in municipal sewage, as well as their concentrations. They choose 50 pharmaceuticals based on their prescribing volumes, their excretion rates, and the type of drug. The model predicted that 29 (58%) of the pharmaceuticals would be present in the influent at concentrations of greater than or equal to 1 µg/l, and 20 (40%) of the pharmaceuticals would still be present in the wastewater at concentrations greater than or equal to 1 µg/l after secondary treatment.

(Carballa *et al.,* 2004) conveyed a study researching the removal efficiency of pharmaceuticals, cosmetics, and hormones in a municipal wastewater treatment plant. The researchers indicated that although during the primary treatment process, target compounds were not removed efficiently (ranged from 20 to 50%), the aerobic activated sludge process caused a significant reduction in all compounds detected, between 30 and 75%, with exception of iopromide, which remained in the aqueous phase. The overall removal efficiencies of wastewater treatment plant could achieve 80% for galaxolide and 83% for onalide; 65% for ibuprofen, 50% for naproxen, approximately 65% for 17β-estradiol, and 60% for sulfamethoxazole.

(Joss *et al.,* 2005; Clara *et al.,* 2005) report no significant elimination of diclofenac. In contrast, (Ternes *et al.,* 1998) observed elimination rates of up to 70%. Removal of diclofenac might partially attribute to the elimination of sludge during primary treatment and an enhanced sorption onto sludge during secondary treatment (log Kd2.7) with an addition of inorganic salts for phosphorus precipitation (Ternes, 1998; Clara *et al.,* 2005a). The biological degradation of diclofenac is very low (<0.1 l g SS^{-1} day^{-1}) (Joss *et al.,* 2006).

(Joss *et al.,* 2005) observed no evident correlation between the compound structure and biological removal efficiency. The observed biological removal varied strongly from compound to compound. Galaxeide were mainly removed by sorption onto sludge with removal deficiency at least 50%. Ibuprofen is often removed beyond the quantification limit (>90%); naproxen also shows significant removal (50–80%). Finally, no removal was seen for Carbamazepine, which consists with other researches (Vieno *et al.,* 2007; Clara *et al.*, 2005).

(Joss *et al.,* 2005; Jones *et al.,* 2006; Nakada *et al.,* 2006; Kreuzinger *et al.,* 2004) confirmed that Ibuprofen exhibits high value of biodegradation kinetic coefficient in range of 20, 9-35, l g SS^{-1} day^{-1}. The hydrophilic nature of this substance makes its sorption onto sludge negligible, which means that the main removal mechanism of ibuprofen is biological degradation and high removal efficiency of ibuprofen (>90%).

Diclofenac is only barely removable during conventional treatment. The removal rates of ibuprofen and naproxen are commonly higher than 75% and 50%, respectively (Castiglioni *et al.,* 2006; Lindqvist *et al.,* 2005). Partial removal was seen for diclofenac (20–40%) and (Clara *et al.,* 2005; Gomez *et al.,* 2007; Joss *et al.,* 2005; Lindqvist *et al.,* 2005) confirmed that the diclofenac shows rather low and very inconsistent removal rates, between 0 and 90%. Its

persistence is attributed to the presence of chlorine group in the molecule. The elimination of beta-blockers in WWTPs depends on the HRT, which could be a good explanation for the variable removal reported in the literature. The highest average elimination rates can be observed for atenolol and sotalol (around 60%). But for the same compounds low removals were reported as well. (Maurer *et al.,* 2007; Wick *et al.,* 2009) reported removal rates lower than 30% for sotalol, and (Castiglioni *et al.,* 2006) reported a removal of 10% foratenolol during the winter months. Concerning hormones, the removal efficiencies of estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) varied strongly between studies. Different kinds of behaviours were observed. (1) (Carballa *et al.,* 2004) observed an increase along wastewater treatment plant. (2) (Clara *et al.,* 2005) indicated that when SRT was higher than 10 days, natural estorgens could achieve around complete removal.

(Nakada *et al.,* 2006) observed a high removal rate (80%) of estrone. High removal efficiencies reported for E1, E2 and EE2 in activated sludge treatment were observed in the range of 49–99%, 88–98% and 71–94%, respectively (Andersen et al., 2003; Joss et al., 2004). (3) In contrast, (Ternes *et al.,* 1999) indicated that there was no significant removal of hormones. High removal rates were observed with increasing SRT, and the trend was most obvious for ibuprofen, bisphenol A and estrogen. A possible explanation for the high removal rates of ibuprofen is elimination in the form of metabolization of hydroxyl Ibrofen and carboxyl-ibuprofen (Clara *et al.,* 2004).

(Castiglioni *et al.,* 2006) found that the removal of ranitidine depends on season, and showed 39% removal in winter and84% in summer. High removal of ranitidine during activate sludge treatment (89%) was observed in a study of (Kasprzyk-Hordern *et al.,* 2009). Although there is still no certain research indicating the explanation of these deviations, some observation can still be made (Suarez *et al.,* 2008). (1) Different temperature of operation might influence removal efficiency (Ternes *et al.,* 1999; Brown *et al.,* 2006; Paxeus et *al.,* 2004; Gros *et al.,* 2006) reported that the removal of these antibiotics has been reported to vary significantly. Due to their limited biodegradability and sorption properties, sulfonamides and trimethoprim appear to be only partially removed by conventional wastewater treatment. In case of trimethoprim, only minor removal was noticed during primary and biological treatment, but the advanced treatment (Gobel *et al.,* 2007) and

nitrification organisms appear to be capable of degrading trimethorpim (Batt *et al.,* 2006; Perez *et al.,* 2005). Also macrolide antibiotics are often incompletely removed during biological wastewater treatment. Studies from different conventional WWTPs have revealed that the removal of macrolides varied from high but negative values, to around 50% (Gobel *et al.,* 2007; Clara *et al.,* 2005). Varying removal was reported for fluoroquinolone antibiotics, as well. (Lindberg *et al.,* 2006) found that norfloxacin and ciprofloxacin were removed with 78% and 0%, respectively, where around 40% was removed during the biological treatment. Ciprofloxacin and norfloxacin sorbed to sludge independently of changes in pH during wastewater treatment, and more than 70% of the total amount of these compounds passing through the plant was ultimately found in the digested sludge (Lindberg *et al.,* 2006).

According to (Suarez *et al.,* 2008): the PPCPs could be distinguished into four categories according to the kbiol and Kd values, and the behaviours of these compounds in biological treatment:

1) Compounds with high kbiol and low Kd values, such as ibuprofen, could be almost totally removed through biological degradation, regardless of SRT and HRT.

2) Compounds with low kbiol and low Kd values, such as carbamazepine, could not be removed nor bio transformed independently of SRT and HRT.

3) Compounds with high kbiol and medium Kd values, such as E2 and E1, are moderately transformed slightly dependant on SRT.

4) Compounds with low kbiol and high Kd values, such as musk fragrances, are removed in the aeration tank by sorption and significantly transformed by biological degradation when the SRT is long enough >10 days).

2.2.2. Membrane Bioreactors (MBR)

The Membrane Bioreactor combines the biological activated sludge process with a membrane filtration step for sludge water separation. The membrane can be applied within the bioreactor (submerged configuration) or externally through recirculation. Since external settlers, or any other post treatment step, become super fluous by using a membrane for the suspended solid and effluent separation, the required space for an installation is small and sludge concentration in the aeration tanks can be two to three times higher than in conventional systems. Furthermore, the effluent quality is significantly better as all

suspended and colloidal material such as micro contaminants, bacteria and viruses are removed (Ujang and Anderson, 2000; Trussell *et al.,* 2005). In an MBR, biological processes are often comparable or better than in conventional activated sludge systems (Ujang *et. al.,* 2005 a, b and c). Due to the long sludge ages, N-removal is more efficient because the slow growing autotrophic bacteria are kept efficiently in the system. Denitrification can occur by introducing anoxic tanks or intermittent aeration (Drews *et. al.,* 2005; Gander *et. al.,* 2000). (Figure 5) shows a typical MBR system.
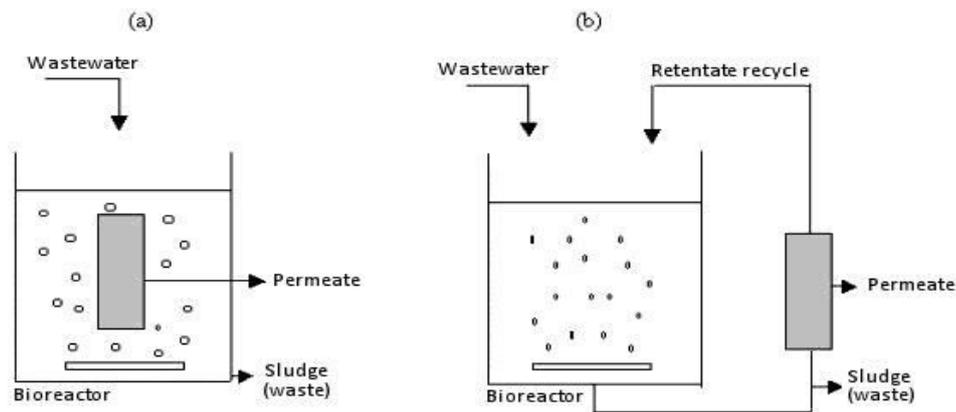
Figure 5: Typical membrane bioreactor system (Pombo *et al.,* 2011)

Membrane filtration denotes the separation process in which a membrane acts as a barrier between two phases. In water treatment the membrane consists of a finely porous medium facilitating the transport of water and solutes through the membrane. It can be also defined as a material that separates particles and molecules from liquids and gaseous. The membrane separation process is based on the presence of semi permeable membrane. The principle is quite simple: the membrane acts as a very specific filter that allows water to flow through, while it catches suspended solids and other substances (Figure 6). There are two factors that determine the effectiveness of a membrane filtration process; selectivity and productivity. Selectivity is expressed as a parameter called retention or separation factor, while productivity is expressed as a parameter called flux.
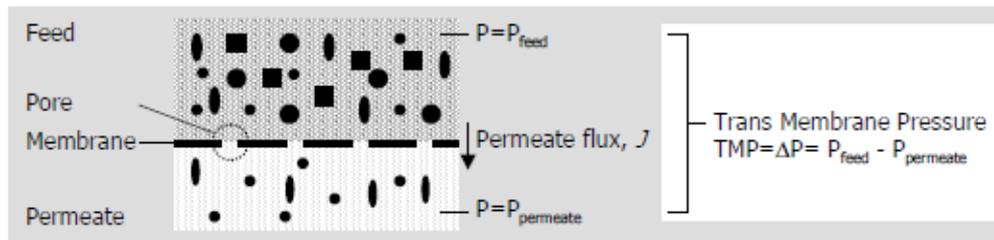
Figure 6: Schematic shapes for membrane filtration process

2.2.2.1. Types of Membrane Modules

Membranes can be configured into membrane modules in different ways. Depending on the production process the membrane can be in the form of sheets, hollow fibres and tubes (Mulder, 1996). Flat sheet membranes are used to construct spiral wound modules or they can be mounted on a frame, resulting in the plate and frame modules (Figure 7). Tubular membranes are usually anisotropic membranes with the separating layer at the inside. Hollow fibre membranes are often isotropic membranes that can be operated inside out or outside in. Submerged hollow fibres can be oriented horizontally or vertically; for application in MBR where air scouring is applied, vertical orientation seems favourable (Chang and Fane, 2000) For the treatment of suspensions flat sheet, tubular and capillary membranes (hollow fibres) are preferred, see also Table 3. In recent years, membrane processes have found wide application and nowadays membrane processes exist for most of the fluid separations encountered in industry, (Bowen and Jenner, 1995).
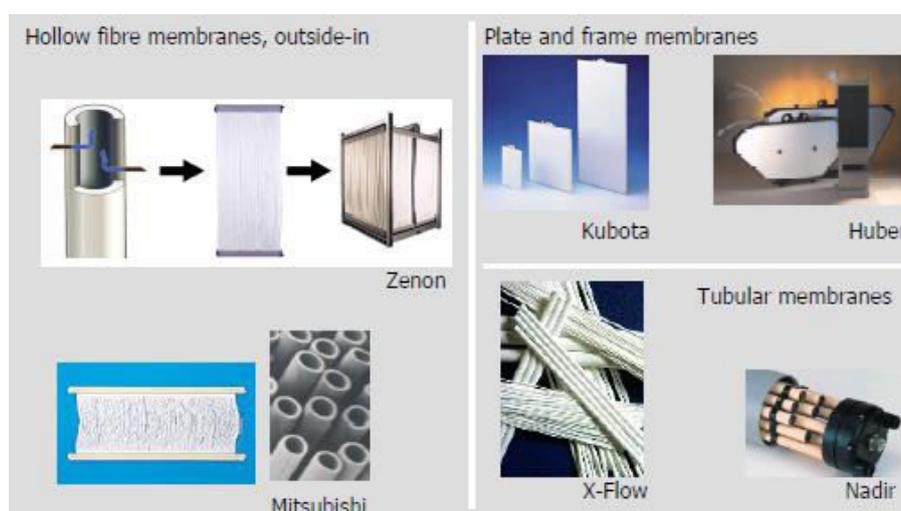
Figure7. Examples of commercially available membranes, applied in cross flow filtration (EVENBLIJ, 2006)

Table 4: Membrane configurations and application in different separation processes (Baker, 2000)

Membrane configuration	Applied in:			
	RO	NF	UF	MF
Spiral wound	x	x	x	
Tubular		x	x	x
Hollow fibre Inside - out				
Hollow fibre outside- in			x	x
Plate and Frame			x	x

Membrane materials can be organics (polyethylene, polyethersulfone, polysulfone, polyolefin, etc), inorganic (ceramic) or metallic and they should be inert and non-biodegradable. Membrane materials should also be easily cleaned and withstand to cleaning chemicals, high temperature and pressure. Moreover, membrane surface must be neutral or negatively charged to avoid adsorption of microorganisms (Seung, 2004).

2.2.2.2. MBR Types

According to its position, MBR system can be classified into two major groups: internal (submerged) and external. Based on the electron acceptor for the biological reaction, the MBR system can be classified into two groups: aerobic and anaerobic MBR (Seung, 2004). The first group is the internal (submerged) MBR, in which membrane filtration unit is integrated into biological reactor to treat and separate biomass (Engelhardt *et al.*, 1998). Recently, this type of MBR has become a promising alternative to the conventional treatment, thus it has been developed to simplify the system and reduce the operational cost (Darren *et al.*, 2006). The driving force across the membrane in the submerged MBR is achieved by creating a negative pressure on the permeate side of the membrane unit (Yamamoto *et al.*, 1989 and Chiemchaisri *et al.*, 1993). Despite, the popularity of submerged MBR and its capability to treat high strength wastewater, it is still prevented by the obstacle of membrane fouling, which causes declining permeate flux and increasing MBR operation costs.

The second group, is the external MBR, in which, the mixed liquor is recirculated through a membrane filtration unit. The driving force in external MBR is the pressure obtained by high cross flow velocity through the membrane filtration unit. Although, the high cost of mixed

liquor recirculation in external MBR, higher effluent fluxes, easier maintenance and less complicated configuration make it desirable (Seung, 2004). Figure 4 (pombo *et al.*, 2006), simply shows the two types of MBR.

A. Aerobic MBR

The aerobic MBR has been applied quite widely to domestic, municipal wastewater treatment instead of the conventional activated sludge system (Gander *et al.,* 2000; Jefferson *et al.,* 2000; Ueda and Hata, 1999 and Murakami *et al.,* 2000). (Darren *et al.,* 2005) reported that, their laboratory-scale aerobic MBR system managed to remove 98% of the suspended solid and achieving a remarkable COD removal efficiency of 96% in treating high strength synthetic wastewater. However, phosphorus removal in MBR varied from 12% (Cote *et al.,* 1997) to 74% (Ueda and Hata, 1999). The concentration of the MLSS is reported to be 10 g/l in some analyses.

B. Anaerobic MBR

Although, the disadvantages of anaerobic MBR such as lower growth rate, high MLSS concentration requirement and long HRT to prevent the biomass from washout, there are advantages of the anaerobic MBR over the aerobic one, which are biogas recovery, lower sludge production and lower energy consumption regarding to the absence of aeration process (Seung, 2004). Although, anaerobic MBR to date is less explored than aerobic MBR, it is a promising system for different strength wastewater treatment with simultaneous energy recovery and less excess sludge production (Dongen *et al.,* 2004).

2.2.2.3. MBR Performance in treating the organic pollutants

Low sludge production was observed in the MBR processes because of limited energy source (Witzig *et al.,* 2002), mechanical shear caused by pumping (Choo and Lee, 1996; Kang *et al.,* 2002; Kim *et al.,* 2001), or attachment on to the surface of membrane (Choo and Lee, 1996). In addition, the sludge age also influences the biomass production. Chaize and Huyard (1991) demonstrated that sludge production was greatly reduced if the sludge age is between 50 and 100 days. The performance of MBR process has been shown to be satisfactory for at least two months when the sludge is completely retained (Chiemchaisri *et al.,* 1993). However, it is unclear if the accumulation of inert material has a negative effect on the

treatment performance. The ratio of VSS to total suspended solids (TSS) in MBR MLSS was reported in the range of 0.46 – 0.55 (Seung, 2004), which is much lower than the 0.75 – 0.90 observed in activated sludge MLSS.

The MBR system is capable of achieving COD removal by both physical and biological mechanisms. The biological COD removal occurs in the bioreactor. The biological COD removal efficiency can be calculated from the difference of soluble CODs in the feed and the mixed liquor divided by soluble COD in the feed (Ng *et al.,* 2000). The membrane filter offers the physical barrier against particulates and some soluble organic carbon and inert fractions of the mixed liquor (Chang *et al.,* 2001). The biological COD removal increases with time, but the physical COD removal by membrane decreases over time because of the age of the membrane and sloughing of some biomass on permeate side of the membrane (membrane fouling). Chang *et al.* (2000) proposed the mechanisms of COD removal by membrane to be due to three mechanisms; sieving method depending on membrane pore size and cutoff, adsorption into membrane pores and surface, and sieving and/or adsorption onto the cake layer, because of this physical removal, the COD concentration in the effluent become lower (Chaize and Huyard, 1991). The difference between the membrane permeate COD and the mixed liquor soluble COD indicates that a fraction of soluble COD components, probably microbial metabolic matter with a relatively large molecular weight, could be removed by the membrane together with biomass (Seung, 2004). The COD concentration also can be reduced by gas production under anaerobic conditions (Anderson *et al.,* 1986; Choo and Lee, 1996; Kang *et al.,* 2002). The changes in HRT and SRT do not significantly influence the COD removal in the MBR. The effect of high temperature on the removal efficiencies in MBR was studied by (Zhang *et al.,* 2006). (Wen *et al.,* 2004) investigated the performance of a submerged membrane bioreactor for treatment of a hospital wastewater. The bioreactor was operated at the conditions of 7.2 h HRT, NH_4^+-N with average 17.7 mg/L and COD range from 49 to 278 mg/L. They found that the removal efficiency for COD, NH_4^+-N and turbidity was 80, 93 and 83%, respectively. The bacteria removal was greater than 98% and the effluent had no colour and odour.

The high nitrification can be observed in the aerobic MBR because membrane separation entirely confines the nitrifying bacteria within the bioreactor independent of sludge concentration. In addition, as sludge production is low in MBR, nitrifying bacteria face less

competition from heterotrophic bacteria, which also consume ammonia. (Cote *et al.,* 1997) reported that ammonia removal efficiency was improved by increasing the sludge age from 10 days to 50 days. (Xing *et al.*, 2002) observed a high nitrification rate at 3.75 hours of HRT and 5 days of SRT.

2.2.2.4. Membrane Operation Parameters

Transmembrane pressure, flux and resistances

The transmembrane pressure is the driving force behind the filtration process. (Equation 3) can be used to predict the permeate flux that remains proportional to hydraulic resistance for porous membrane system. The flux is the quantity of materials passing through a unit area of membrane per unit time and can be determined by both the driving force and the interfacial region adjacent to it. Under the simplest operating conditions, the resistance to flow is offered entirely by the membrane.

$$J = \frac{\Delta P}{\mu R_t} \qquad\qquad \text{Eq. 3}$$

Where J: permeate flux (L/m2.h)

ΔP: transmembrane pressure (kPa)

μ: Viscosity of the permeate (Pa.s); (For example; Viscosity at 30ºC = $0.798*10-3$ N.s/m2) when Pa = N/m2

R_t: total resistance (1/m): $R_t = R_m + R_c + R_f$

R_m: intrinsic membrane resistance

R_c: Cake resistance from by the cake layer (reversible fouling)

R_f: fouling resistance caused by solute adsorption into the membrane pore and gel formation (irreversible fouling).

All resistances shown in Equation 2.1 can be measured through a series of filtration experiments by comparing pure water filtration, sludge filtration, and pure water filtration after cake removal. However, the resistances are dependent on a number of experimental conditions, such as biomass characteristic, membrane material and temperature. (Figure 8) shows a relationship between transmembrane pressure and flux. It is observed that the

higher the transmembrane pressure and the flux, the faster the particles deposit on the membrane surface and to form a cake, then the flux is independent of the transmembrane pressure and remains constant. (Günder, 2001)
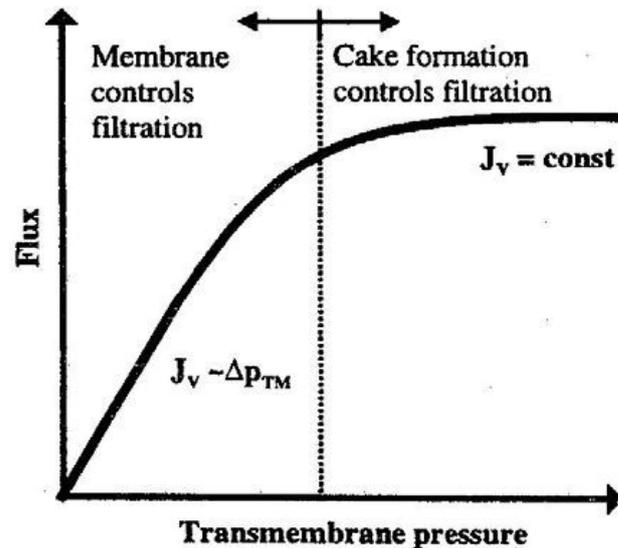
Figure 8: Relationship between transmembrane pressure and flux (Günder, 2001)

2.2.2.5. Membrane fouling

Several definitions of fouling can be found in literature. A broad definition is given by (Cheryan, 1998) 'Fouling manifests itself as a decline in flux with time of operation, and in its strictest sense the flux decline that occurs when all operating parameters (…) are kept constant'. Lojkine and coworkers also leave out short term phenomena, and define it somewhat different: 'Fouling is a blanket term used to cover the physicochemical causes of flux decline, which are NOT reversed when the transmembrane pressure is relaxed' (Lojkine *et al.,* 1992).   Different fouling mechanisms may occur during cross flow membrane filtration, some of which were mentioned before (Figure 9) (vanden Berg and Smolders, 1990):
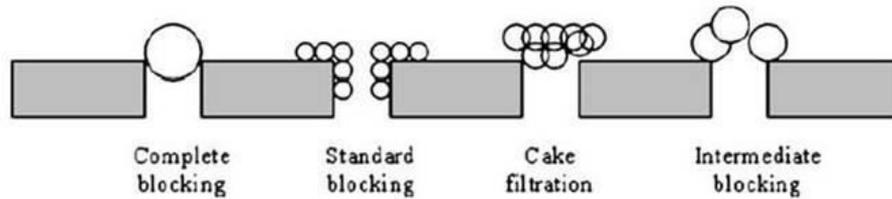
Figure 9. Fouling mechanisms in a membrane filtration (Radjenovic *et al.,* 2008)

➢ Pore blocking

Particles enter the pore and get stuck in its opening, reducing the number of pore channels available for permeation.

➢ Pore narrowing, e.g. by adsorption. Substances and/or particles enter the pores and are adsorbed to the pore wall, thus narrowing the pore channel, reducing the permeate flow.

➢ Gel or Cake layer formation. Particles and macromolecules accumulate at the membrane surface, forming a more or less permeable layer. When its constituents are non-interacting, the cake layer may disappear when TMP is released or cross flow in increased. If there is an interaction the particles may form a cohesive gel layer, which is difficult to remove. In both cases the fouling mechanism will lead to an increase in total filtration resistance.

Since suspended solid are totally eliminated through membrane separation, the settle ability of the sludge, which is a problem in conventional activated sludge, has absolutely no effect on the quality of the treated effluent. Consequently, the system is easy to operate and maintain.

The major advantages of the membrane separation bioreactors are:

➢ Sludge retention time (SRT) is independent of hydraulic retention time (HRT). Therefore a very long SRT can be maintained resulting in complete retention of slowly growing microorganisms, such as nitrifying bacteria, in the system.

➢ The overall activity level can be raised since it is possible to maintain high concentrations in bioreactors while keeping the microorganisms dispersed as long as desired and as a result, reactor volume will be reduced. In addition, the system requires neither sedimentation nor any post-treatment equipment to achieve reusable quality water, so the space saving is enormous.

➢ Treatment efficiency is also improved by preventing leakage of undecomposed polymer substances. If these polymer substances are biodegradable, there will be no endless accumulation of substances within the treatment process. On the other hand, dissolved organic substance with low molecular weights which cannot be eliminated by membrane separation alone can be taken up, broken down and gasified by microorganisms or converted into polymers as constituents of bacterial cells, thereby raising the quality of treated water.

2.2.2.6. Membrane Bioreactors application for treating the hospital wastewater

The use of Membrane Bioreactors (MBR) in hospital wastewater treatment has grown widely in the past decades. The MBR technology combines conventional activated sludge treatment with low-pressure membrane filtration, thus eliminating the need for a clarifier or polishing filters. The membrane separation process provided a physical barrier to contain microorganisms and assures consistent high quality reuse water. Few studies was found in the literature explained the efficiency of MBR in treating the hospital wastewater and removal the pharmaceutical s compounds. The wastewater treatment technologies analyzed included microfiltration, ultrafiltration, Nanofiltration, granular activated carbon, powdered activated carbon, reverse osmosis, electro dialysis reversal, membrane bioreactors, and combinations of these technologies in series.

Microfiltration was not shown to be effective at removing the majority of organic compounds tested. However, microfiltration did effectively remove steroids, especially when coupled with a membrane bioreactor.

Ultrafiltration reduced concentrations but was not shown effective at removing the majority of organic compounds tested. However, ultrafiltration effectively removed steroids, especially when coupled with a membrane bioreactor. Snyder et al. (2006a) determined that ultrafiltration provided an average removal rate of 59%, and ranged from 1% to 100% depending on the chemical.

Nanofiltration was shown to be capable of removing almost all the pharmaceuticals tested, although a few pharmaceuticals were present in the outlet.

(Clara and co-workers, 2005) found that diclofenac removal by size exclusion failed, but a partial removal could be obtained by rising the sludge retention time. Ibuprofen on the

other hand was removed to a high degree (> 90%). Carbamazepine was not removed at all. In contrast to (Clara *et al.,* 2005 a) study by (Radjenovic *et al.,* 2007) indicated a better pharmaceutical removal in some cases (Diclofenac removal of 87.4% in MBR compared to 50.1% in conventional treatment; Metoprolol removal 58.7% compared to 0%; Clofibric Acid removal 71.8% compared to 27.7%), compared to conventional treatment. But again, some treatment results were similar to conventional treatment (e.g. for Ibuprofen, removal > 80%) and carbamazepine passed both systems without degradation or transformation. For sulfamethoxazole a variation of removal rates were found. Maybe there was some back conversion of the human metabolite N4-acetylsulfamethoxazole to the initial compound during treatment. Interestingly, the membrane pore size of 0.4 µm decreased during operation of the MBR to a size of 0.01 µm due to microbial fouling.

(Sipma *et al.,* 2010) confirmed that it can be seen that easily removed pharmaceuticals are equally well removed in both systems, i.e. acetaminophen, ibuprofen and paroxetine. Nevertheless, in most cases the removal efficiency of moderately or slightly removed pharmaceuticals in CAS is better in an MBR, although the removal efficiency inmost cases are far from complete. In some cases, i.e. sotalol and hydrochlorothiazide the removal efficiencies reported in an MBR were worse than the reported values in a CAS. It should be noted that for some pharmaceuticals the number of different treatment facilities analyzed is rather limited.

(Snyder *et al.,* 2007) reported that concentrations of caffeine, acetaminophen, sulfameth-oxazole, carbamazepine, and gemfibrozil decreased as the compounds passed through the pilot MBR with removal efficiencies varying between 99.1% (sulfamethoxazole) and 99.9% (acetaminophen). (Radjenovic *et al.,* 2009) found that the removal of acetaminophen from the aqueous phase by the MBR was greater than 99% (similar to the CAS). No elimination of gemfibrozil took place by CAS treatment, whereas 30-40% of this compound was eliminated by the MBR. In the same study, carbamazepine remained untreated by both technologies. Removal efficiencies of sulfamethoxazole were higher by the MBR technology (81%) than by the conventional activated sludge (75%).

(Kimura *et al.,* 2005) investigated the ability of submerged MBR at a municipal WWTP to remove six pharmaceuticals and one herbicide (dichlorprop). They compared this treatment to the removal efficiency of an activated sludge process. The MBR demonstrated a better removal rate for ketoprofen and naproxen. For the other compounds, the removal rate was comparable with activated sludge. The authors attributed the poor removal of some compounds in both treatment processes to either the inclusion of chlorine within their chemical structure, or a double aromatic ring structure. Ibuprofen has a relatively simple chemical structure with no chlorine molecules, and both treatment systems efficiently removed it.

(Bernhard *et al.,* 2006) reported that treatment by MBR resulted in significant better removals compared to activated sludge treatment for poorly biodegradable persistent polar pollutants, such as diclofenac, mecoprop and sulfophenylcarboxylates, which was ascribed to the employed long sludge retention times.

Also (Radjenovic *et al.,* 2009) reported that the removal of pharmaceuticals in an MBR was superior for several compounds and at least similar for others, and furthermore, that the range of variation of the removal efficiency in the MBR system was smaller for most of the compounds. Obviously, non-degradable micro-pollutants, such as EDTA and carbamazepine were not eliminated at all by any treatment process (Bernhard *et al.,* 2006).

(Kimura *et al.,* 2005) found that compounds with a complex chemical structure, e.g. ketoprofen and naproxen, were not eliminated in a CAS treatment process, but could be eliminated by a MBR. These authors found further that for several other pharmaceuticals the treatment efficiencies were comparable. Others have also reported on similar removal efficiencies between MBR and CAS (Joss *et al.,* 2005; Clara *et al.,* 2004).

In general MBR technology generally outperforms the CAS treatment in removing PhCs from WWTPs (Radjenovic *et al.,* 2009). From the aspect of the excess sludge produced, advanced MBR technology would be attractive concept, not only in terms of the cost reduction of sludge treatment due to its lowered production, but also because it diminishes the environmental impact of WWTPs treatment, since the MBR sludge is less contaminated with PhCs than the sludge produced during the conventional treatment. The amount of PhCs,

sorbed onto sewage sludge may increase the environmental risk of these micropollutants, since they can become bio available when conditions for desorption are created.

Moreover the literature in (Bouju *et al.,* 2008) shows that MBRs should be more efficient on Persistent organic pollutants (POPs) removal than CAS, especially on the substances which are poorly biodegradable, while it does not improve the removal efficiency for the non-degradable ones. The comparison with the removal obtained in a very large conventional WWTP operating at quite high SRT will be particularly significant. Also (Hawkshead, 2008) concludes that MBR system can represent an important alternative to CAS in the HWWs treatments and, for a correct treatment of HWWs, (Beier *et al.,* 2011) reports the design requirements for MBRs. Based on the operational experience gained at this site and on technical and economic optimisation, the following aspects should be considered in the design of MBR treating hospital wastewaters in high density urban areas:

➢ Separate rainwater collection to reduce dilution effects

➢ Where applicable, separation of water streams with low pharmaceutical concentrations (e.g. kitchen and laundry wastewaters) sludge age in the MBR > 100 days to allow for biomass adaptation

➢ Thermal treatment of the waste activated sludge and screenings for complete destruction of the adsorbed pharmaceuticals

➢ Consideration of the special requirements on emission levels (noise and aerosols) for hospital patients with a weak immune system and/or needing a quiet environment as well as those of nearby residents.

Membrane Biological Reactors (MBR) have gained significant popularity in STPs and are nowadays considered as a powerful (and expensive) technology able to produce higher quality effluents in terms of conventional pollutants, which can be appropriate for direct discharge, further postreatment or even reuse purposes. However, since membrane filtration does not enhance the elimination of most micropollutants by means of a size-exclusion mechanism it is still not clear if these systems may effectively enhance the removal of organic micropollutants (Reif *et al.,* 2008).

The need for compact wastewater treatment plants increasingly becomes a global concern where the environmental impact by the population also sets high demands to treatment of waste produced by the community as the hospital wastewaters. The attached growth bioreactor coupled with membrane separation as attached growth membrane bioreactor

(attached growth MBR) is an alternative way to achieve high effluent quality, compactness treatment plants and economical management (Ødegaard, 2000).

2.2.3. Attached growth biological treatment technology

The removal of organic micropollutants from wastewater has become an increasingly important consideration and has imposed new challenges in the design of wastewater treatment plants. One such technology is the submerged attached growth bioreactor (SAGB), which derives its name from the fact that the media is always submerged in the process flow. Attached growth technologies work on the principle that organic matter is removed from wastewater by microorganisms. These microorganisms are primarily aerobic, meaning they must have oxygen to live. They grow on the filter media (materials such as gravel, sand, peat, or specially woven fabric or plastic), essentially recycling the dissolved organic material into a film that develops on the media. The two primary advantages of a SAGB are the small volume requirement and the elimination of downstream clarification (Grady *et al.,* 1999). A submerged biofilter allows for a high biomass concentration leading to a short hydraulic retention time and, thus, a significantly reduced reactor volume as compared to a different fixed film reactor or a suspended growth reactor. In addition, the media in a SAGB may be fine enough to provide physical filtration for solids separation.

Attached growth aerobic treatment reactors can be divided into two groups: with up flow and down flow of treated water. Up flow attached growth aerobic treatment reactors differ in the type of packing and the degree of bed expansion. Down flow attached growth reactors differ only in the packing material used and these can be random or tubular plastic (figure 10). The neutrally plastic media within each aeration tank provides a stable base for the growth of a diverse community of microorganisms. Polyvinylchloride (PVC) media has a very high surface-to-volume ratio, allowing for a high concentration of biological growth to thrive within the protected areas of the media.

There are three types of up flow attached growth processes: 1) the up flow packed bed reactor, where the pack material is fixed and the wastewater flows between the packing covered by the biofilm. The packing material can be rock or synthetic plastic. 2) The aerobic expanded bed reactor (AEBR) which uses a fine-grain sand to support the biofilm growth. 3) The

fluidized-bed reactor (FBR), in which fluidization and mixing of the packing material occurs. (Tchobanoglous, 2003).
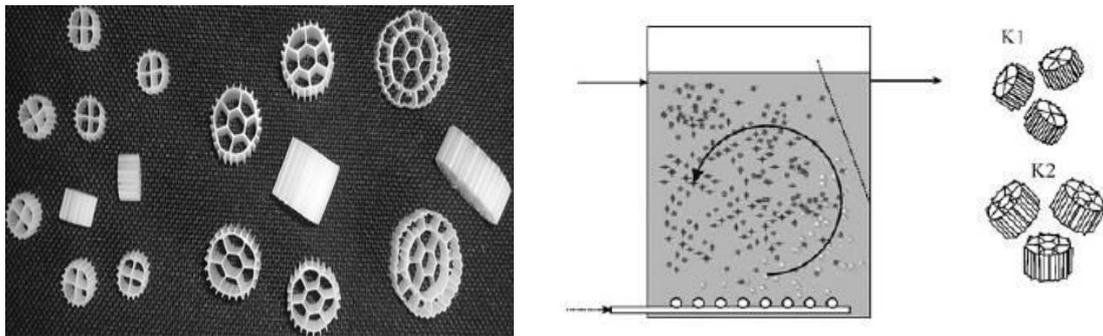
Figure 10: Photo of (from left to right) Kaldnes type K1, K2 and K3 biofilm carriers and schematic of the moving-bed-biofilm reactor (MBBR). (Rusten *et al.,* 1994; Leiknes and Ødegaard, 2007)

The main advantages of attached growth processes over the activated sludge process are lower energy requirements, simpler operation, no bulking problems, less maintenance, and better recovery from shock loads (Metcalf and Eddy, 2003). Attached growth processes in wastewater treatment are very effective for biochemical oxygen demand (BOD) removal, nitrification, and denitrification. Disadvantages are a larger land requirement, poor operation in cold weather, and potential odour problems.

Attached growth processes technology for optimum performance and dependability. Using reliable, cost effective and energy efficient blower for aeration are with an integral flow management system and enter the biological treatment stage where it is aerated with fine bubble membrane diffuser. The continuous supply of oxygen together with the incoming food sources encourage microorganism to grow on the surface of the submerged media, convening the wastewater in to CO_2 and water in the process. Media of SAFF is providing more surface area for microorganism to grow. Excess micro-organism that flows out of the biological treatment stage is separated from the final effluent in another settlement stage. (Jafrudeen *et al.,* 2012).

In wastewater treatment processes, development of attached growth bioreactor with high biomass concentrations has been of interests to be achieved in short hydraulic retention time (HRT) in comparison to suspended growth system with equivalent solid retention time (SRT). This results from the use of high specific surface area of carriers. Short HRT could lead

to a compact system of the reactor, which can be beneficial when the plant area is limited. (Comett *et al.,* 2004) studied a treatment of leachate wastewater from the anaerobic fermentation of solid wastes using two biofilm support media. Biofilm growing on different carrier media had different responses to the nutrient contaminated in wastewater. The sequencing batch system consisted of two reactors containing Kaldnes and Linpor carrier materials with specific areas of 490 and 270 m2/m3, respectively. The total COD removals for Linpor and Kaldnes reactors were 47% and 39%, respectively and the average ammonia removals for Linpor and Kaldnes were 72% and 42%, respectively. The surface of Linpor had higher concentrations of microorganisms than that of Kaldnes. The average dry solids in Linpor and Kaldnes were 170 g/m2 and 63 g/m2, respectively.

2.2.3.1. Application of attached growth biofilms with membrane bioreactors

The typical attached membrane bioreactor consists of a bioreactor with a membrane module and media submerged in the bioreactor. There are blowers in the bottom of the bioreactor to supply air for the biomass and suspend the media in the bioreactor. The media are used to collide the membrane surface to reduce the thickness of cake layer. On the other hand, the biomass could attach on the surface of media to increase the biomass concentration and reduce the sludge production. So, the attached membrane system does not required large space, since clarifier is not need in the system. Also, retaining relatively high biomass concentration in attached membrane system over MBR or attached system would increase removal efficiency and retain nitrifiers for increasing nitrification. The lab scale attached membrane bioreactor is showed in (Figure 11).
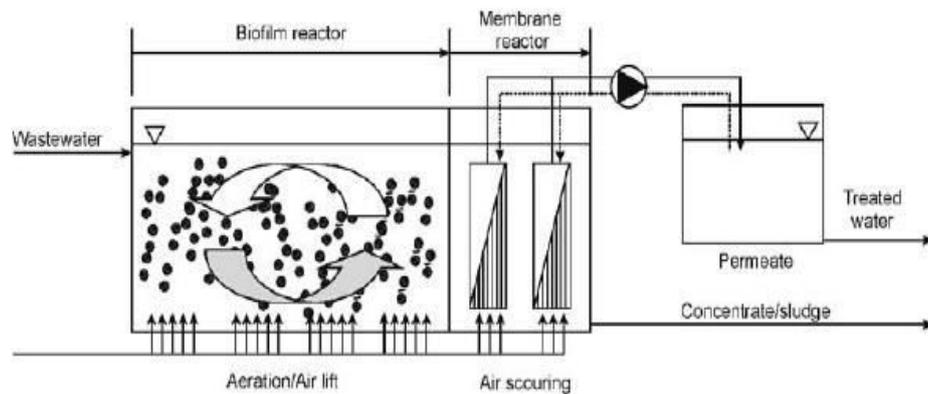
Figure 11: Typical schematics of a attached membrane bioreactor (Lee *et al.,* 2001; Leiknes and Ødegaard, 2007)

(Lee *et al.*, 2001) reported filtration performance between attached and suspended growth systems in a submerged membrane bioreactor (MBR) under comparable operating conditions. Hollow fiber membrane with pore size 0.1µm was immerged in the bioreactor and the reactors were fed with synthetic wastewater at a constant flux of 25 L/m2.d. For the attached growth MBR (see figure 10), looped core media (BioMatrix®) of the total surface area 4.37 m2 was immerged into the reactor. Suspended growth MBR was set up and operated at the same conditions with attached growth, except for the elimination of the looped media from the bioreactor. The performance of MBRs was determined in terms of filtration characteristics and quality of treated water. The treatment efficiencies of both reactors were greater than 98% of COD and 95% of NH_4 removals under 8 h HRT. The rate of fouling was evaluated by an increasing in transmembrane pressure (TMP). The increasing rate of TMP for the attached growth MBR was 7 times higher than that for the suspended growth MBR. Better filtration performance with the suspended growth was explained by the formation of dynamic membranes with the suspended solids. The suspended growth had smaller specific cake resistance due to the rougher cake layer than that with the attached growth. (Leiknes and Ødegaard, 2001) investigated a potential of membrane separation unit combined with a high-rate moving-bed-biofilm reactor for the design of compact wastewater treatment plants as shown in Figure 2.6. The loading rates used were in the range of 30 to 45 kg COD/ m3.d with HRT of 20-30 min. The results showed 85-90% of COD removal efficiency if the biomass and particulate COD were completely removed in the moving bed reactor. Membrane separation of the biomass and particulate COD was maintained with a constant flux of 60 L/m2.h and showed a high permeate quality in terms of suspended solid of less than 5 mg/L and turbidity of less than 1 NTU. Compared to other membrane bioreactors, the

moving bed biofilm reactor could operate at higher volumetric loading (10-15 times) and at shorter HRT (10-30 times).

(Snyder et al., 2007a) performed a comprehensive analysis of the use of various membrane and activated carbon technologies on the removal of pharmaceuticals, endocrine-disrupting compounds, and personal care products.

2.2.4. Activated carbon adsorption

Activated carbon is a solid, porous, black carbonaceous material, (see Figure 12). It is distinguished from elemental carbon by the absence of both impurities and an oxidized surface (Mattson and Mark, 1971).
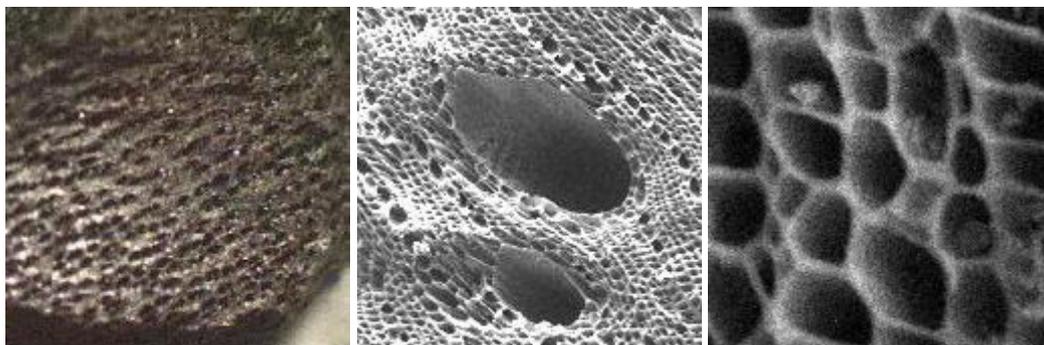
Figure 12: Activated carbon: surface and pores – scanning electron microscope image. Magnification increases from left to right. (Courtesy of Roplex Engineering Ltd.).

Activated carbon has an extraordinarily large surface area and pore volume, making it suitable for a wide range of applications. The dynamics of adsorption in a packed activated carbon bed are influenced by the shape and size of the activated carbon particles and their effect on the flow characteristics. The smaller an activated carbon particle is, the better the access to its surface area and the faster the rate of adsorption. For spherical beads, the diameter can be measured easily. For cylindrical extrudates, an equivalent spherical diameter, d eqv, can be calculated from the radius and length of the extrudate. However, for particles of irregular shape and a wide size distribution, it is difficult to derive d eqv. In such cases particle sizes derived from sieve analyses can be useful parameters for determining adsorption rate.

The most important property of activated carbon, the property that determines its usage, is the pore structure. The total number of pores, their shape and size determine the

adsorption capacity and even the dynamic adsorption rate of the activated carbon. IUPAC classifies pores as follows (Rodriguez-Reinoso and Linares-Solano, 1989):

➢ macropores: $d_0 > 50$ nm

➢ mesopores: $2 \leq d_0 \leq 50$ nm

➢ micropores: $d_0 < 2$ nm

➢ ultramicropores: $d_0 < 0.7$ nm

➢ supermicropores: $0.7 < d_0 < 2$ nm

Where: d_0 is the pore width for slit type pores or the pore diameter for cylindrical pores. The macropores act as transport pathways, through which the adsorptive molecules travel to the mesopores, from where they finally enter the micropores. The micropores usually constitute the largest proportion of the internal surface of the activated carbon and contribute most to the total pore volume. Most of the adsorption of gaseous adsorptive takes place within these micro pores, where the attractive forces are enhanced and the pores are filled at low relative pressures. Thus, the total pore volume and the pore size distribution determine the adsorption capacity. The dynamics of adsorption in a packed activated carbon bed are influenced by the shape and size of the activated carbon particles and their effect on the flow characteristics. The smaller an activated carbon particle is, the better the access to its surface area and the faster the rate of adsorption. For spherical beads, the diameter can be measured easily. For cylindrical extrudates, an equivalent spherical diameter, d_{eqv}, can be calculated from the radius and length of the extrudate. However, for particles of irregular shape and a wide size distribution, it is difficult to derive d_{eqv}. In such cases particle sizes derived from sieve analyses can be useful parameters for determining adsorption rate. **(** Jufang Wu**,** 2004)

2.2.4.1. Application of activated carbon

In the water and wastewater treatment, activated carbon is used, single or coupled with another process, either in powdered (suspension process) or granular (fixed bed process), depending upon the specific application and process. The objectives pursued with the use of activated carbon in water treatment have changed significantly in recent decades. Years ago, activated carbon was employed primarily for the removal of excess chlorine and the elimination of substances affecting odour and taste from relatively good-quality raw water. Increasingly exacting quality requirements for drinking water, coupled with increasing

pollution levels in untreated water (groundwater and surface water), have led to the optimization of activated carbon as a means of guaranteeing acceptable drinking water quality. In parallel, changes in treatment processes, such as the reduction of high-strength chorine treatment, have resulted in the elimination of traditional applications.

In recent years, the use of activated carbon processes has become widely established in drinking water treatment, groundwater rehabilitation and the treatment of wastewaters. Likewise, activated carbon is being used to an increasing extent in waste water treatment, whether it be in the systematic treatment of individual effluent streams (e.g. in the chemicals industry or hospital effluents), in the removal of substances toxic to bacteria in biological waste water treatment or in tertiary waste water treatment, where effluent restrictions are particularly severe.

2.2.4.2. Removal of Micropollutants in Activated Carbon

Powdered and Granular Activated Carbon (PAC and GAC) have been commonly used for sorption of organic micropollutants like pesticides or taste and odour compounds (Ternes and Joss, 2006). Several studies have also evaluated the adsorption of other emerging trace organics including a range of PhACs and ECDs on activated carbon in both laboratory systems and full scale drinking water treatment plants (Kim *et al.,* 2010; Ternes *et al.,* 2002). Only a few studies have investigated GAC adsorption as an option for tertiary treatment of conventional biologically treated wastewater.

Previous studies carried out by our group (Serrano *et al.,* 2010) showed that a GAC addition of 0.5e1 $g.L^{-1}$ directly into the aeration tank of an activated sludge reactor can be a useful tool to increase the removal of the recalcitrant PPCPs carbamazepine, diazepam and diclofenac. Moreover, recent works have shown that activated carbon can be useful to minimise fouling problems in MBRs. In this way, the use of PAC concentrations of $0.5 \cdot 10^3$ $g.L^{-1}$ inside the aeration tank of a MBR have been used to attain an easier control of membrane fouling (Remy *et al.,* 2009). However, activated carbon addition is not common in STPs. With activated sludge processes, (Ng and Stenstrom, 1987) showed that the use of 0.5- 4 $g.L^{-1}$ of PAC may enhance nitrification rates by 75 and 97%, whereas other authors observed an improvement of organic matter removal as well as a significant decrease of toxicity caused

by certain inhibitors on the nitrification process (Widjaja *et al.,* 2004). In fact, activated carbon is a suitable support for bacterial attachment, being possible in this way to enhance the retention of the more slowly growing bacteria, such as nitrifiers (Thuy and Visvanathan, 2006; Aktas and Cecen, 2001).

The activated carbon amended MBR to date has been mainly studied in relation to membrane fouling mitigation (Guo *et al.,* 2008) and rarely to assess recalcitrant pollutant removal enhancement(Hai *et al.,* 2008). Only two studies (Zhang *et al.,* 2008; Lee *et al.,* 2011) to date have explored PAC-amended MBR specifically for the removal of micropollutants. Although (Zhang *et al.,* 2008) confirmed improved removal efficiency; a comprehensive understanding of the involved phenomena is yet to be developed. (Remy *et al.,* 2009) proposed a very comprehensive resume of the main research contributions on this issue and reported that the addition of low PAC concentrations can increase the permeate flux of about 10% by improving the membrane filtration performance. (Fang *et al.,* 2006; Ng *et al.,* 2008) indicated the adsorption of foulants to the PAC particles as the responsible mechanism ($2–5$ g·PAC·L^{-1}activated sludge) of fouling reduction, but also observed as frequent refreshing of the PACs was necessary because foulants saturate them, while operation at an infinite solid retention time (SRT) did not exhibit a positive effect on filterability.

Results from these previous experiences reported in technical literature, confirming that activated carbon addition, in low concentrations, makes possible to halve the permeate flux loss while other tests carried out with higher concentrations did not reveal significant efficiency improvements. Moreover, the temperature influence (considering two series of tests carried out at 12 and 22 °C, respectively) is negligible. The activated carbon addition can contribute to reduce the membrane fouling in MBR systems. The benefit of activated carbon addition improves the MBR filtration performances, such as the energy consumption reduction due to mitigation of TMP increase (or flow rate decrease), elongation of cleaning in place as well as physical cleaning intervals

3. Characteristics of activated sludge flocs

In the literature, it is difficult to find specific descriptions of sludge characteristics in MBR systems, and therefore this section will describe sludge characteristics mainly based on the conventional activated sludge processes. Extracellular polymeric substances (EPS) play an important role in the development of this specific matrix and represent a major sludge floc component beside water phase and cells (Li and Ganczarczyk, 1990). In addition, inorganic and organic substances are adsorbed from the water phase to the flocs and hence sorption processes are involved in floc formation and consequently in the elimination process of wastewater pollutants. The structure and composition of activated sludge flocs are very complex and are directly or indirectly related to sludge settleability. The microorganisms in the bioreactor metabolize dissolved and suspended organic components of the feed wastewater and this process is advantageous due to high chemical conversion efficiency (Judd, 2006). The aerobic processes are ideally capable of converting large organic molecules into CO_2, H_2O and inorganic nitrogen products (Judd, 2006). However, some amounts of extracellular polymeric substances (EPS) will also be produced depending on the conditions in the reactor as well as the feed composition (Judd, 2006). The efficiency of the processes and the production of byproducts depend on various operation parameters, both physical and biological (Judd, 2006).

In activated sludge systems filamentous bacteria were often observed and it was suggested that they provide a stabilizing backbone for the three-dimensional floc structure (Bossier and Verstraete 1996). However, extensive growth of filamentous bacteria is often associated with settling problems such as bulking or scum formation. Bulking sludge is characterized by sludge flocs from which filamentous bacteria grow into the surrounding liquid inhibiting formation of dense floc aggregates under low hydrodynamic shear (e.g. during sedimentation). In contrast, scum formation is caused by sludge flocs float to the surface aggregating to a more or less stable sludge layer at the water-air interface. According to (Lemmer and Lind, 2000) three different groups of filamentous bacteria involved in settling problems are frequently found in municipal WWTPs. Sulfur bacteria such as type 021N and *Thiothrix* sp., which are able to use beside organic substrates reduced sulfur components as energy source, and heterotrophic bacteria adapted to high sludge load (F/M ratio > 0.15 kg BOD_5 kg^{-1} MLSS d^{-1}), e.g. *Sphaerotilus* spp. and *Haliscomenobacter hydrossis*, are in general

responsible for bulking sludge. The third group including heterotrophic bacteria adapted to low sludge load (F/M ratio < 0.15 kg BOD_5 kg^{-1} MLSS d^{-1}) is often found in nutrient removal plants with nitrogen elimination. Eukaryotic organisms are also found in activated sludge systems. However, activated sludge does not usually favour growth of fungi because of fungi being selected by extremely low pH values below 4. In contrast, monocellular protozoa comprising flagellates, amoeba, and ciliates, and highly organized metazoa such as rotifers, nematodes, and other worms play an important role in the activated sludge system. The primary role of both protozoa and metazoa is to clarify the effluent by predation on freely suspended bacteria and bacteria loosely attached to the floc surface.

3.1. Floc morphology and composition

(Wilén *et al.,* 2008) confirmed that most of the microorganisms in conventional activated sludge processes self-aggregate in complex sludge flocs, which mainly consist of bacterial colonies surrounded by a network of extracellular polymeric substances (EPS). Besides, the flocs include organic fibres and particles and inorganic components as presented in the (figure 13).

Sludge typically has a bimodal size distribution, and this has been observed in MBR systems as well (Le-Clech *et al.,* 2006).
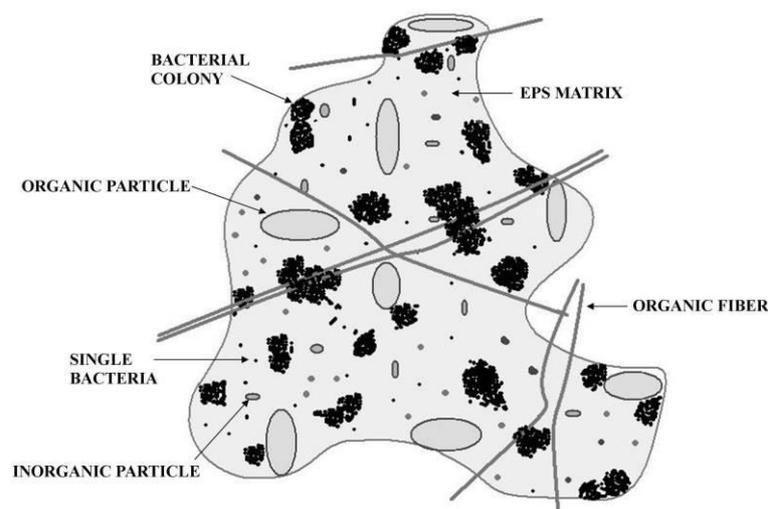
Figure 13: Schematic example of the structure of an activated sludge floc including single bacteria, bacterial colonies, absorbed organic and inorganic particles and organic fibres surrounded by the EPS matrix, Adapted from (Mikkelsen, 1999).

The smaller fraction is primary particles, e.g. single bacteria and colloids, and the larger fraction is the sludge flocs, respectively (Mikkelsen and Keiding ,1999). (Mikkelsen and Keiding, 1999) was demonstrated that the bimodal distribution results from an equilibrium between flocculation and deflocculation i.e. aggregation of new flocs or incorporation of primary particles into existing flocs and erosion of particles from the surface of existing flocs or large scale fragmentation of flocs, respectively (see Figure14). (Jarvis *et al.,* 2005) confirmed that the state of this equilibrium depends on the strength of the forces involved in the interaction within the sludge flocs and the external shear forces applied on the flocs
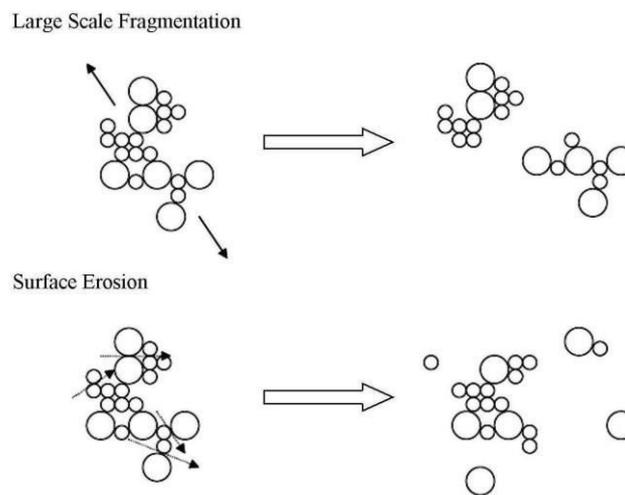
Figure 14: Floc breakage involves either large scale fragmentation or surface erosion, Adapted from (Jarvis *et al.,* 2005).

Floc strength can be regarded as a sum of all the interactions that bind bacteria and floc constituents together. The four most commonly cited floc-binding interactions are the DLVO-type interactions (Hermansson, 1999), bridging of EPS with divalent (Eriksson and Alm, 1991) and trivalent cations (Nielsen and Keiding, 1998), hydrophobic interactions (Urbain *et al.*, 1993), and physical entanglement of floc entities (Rijnaarts *et al.*, 1995). All these interactions can be affected by both physico-chemical properties of bulk liquid and biological activity of bacteria inhabiting the flocs, which makes the floc strength a continuously changing parameter, the magnitude of which can be managed with a number of strategies. According to the DLVO theory, bacterial adhesion to floc surface can be increased by increasing the ionic strength of the solution. This effect is expected to result from decreasing the double layer thickness and decreasing the surface potential, which would eventually act against the electrostatic repulsive forces (Hermansson, 1999). EPS bridging mechanisms are

facilitated by the presence of di- and tri-valent cations, especially calcium, iron and aluminum. However, the reduction of Fe(III) to Fe(II) by anaerobic bacteria (Nielsen, 1996; Nielsen *et al.*, 1997), or Fe(III) precipitation as iron sulphide (Nielsen and Keiding, 1998), results in immediate decrease in floc strength leading to deflocculation and, subsequently, to problems with sludge settling and dewaterability. Hydrophobicity of cells and floc surfaces has been shown to be a very important selective force in a wastewater treatment plant, capable of leaving the hydrophilic species unattached and, as a consequence, removing these species with effluent (Zita and Hermansson, 1997). All these mechanisms are a combination of chemical and microbiological processes and stand between these two worlds. It is therefore very important to remember that any action, designed to interact with one process, will most probably affect other processes, and the overall effect can be different than initially assumed.

The most important component with regards to stability and structure of the sludge floc is EPS, which typically constitute 50 to 60 % of the organic fraction of sludge flocs whereas the cell biomass only constitutes 2 to 20 % of same (Wilén *et al.,* 2003).

3.2. Extracellular Polymeric Substances

EPS matrix of activated sludge flocs constitutes 80 to 90% of organic matter in activated sludge and therefore determines the integrity of flocs to a very high extent (Frølund *et al.,* 1996; Münch and Pollard, 1997; Liu and Fang, 2002). The abbreviation EPS has been used for exopolymers, exopolysaccharides, extracellular polysaccharides, and extracellular polymeric substances. In one of the first reviews EPS was defined as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates" (Geesey, 1982). Another definition for EPS can be found in the glossary to the report of the Dahlem Workshop on Structure and Function of Biofilms in Berlin 1989 (Characklis and Wilderer, 1989): "EPS are organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)". Such biopolymers are synthesized and excreted by bacterial metabolism, and in addition originate from cell lysis. Data from pure cultures support the observation that many bacteria produce a range of EPS (Brown and Lester 1980; Jahn and Nielsen, 1995).

The work of Novak and Park resulted in the fractionation of activated sludge extracellular polymers into three major groups according to the distinct cations responsible for attachment of these polymers: (1) polymers composed of lectin-like proteins bound to polysaccharides, bridged by Ca^{2+} and Mg^{2+} and extractable by a sodium-rich cation exchange resin (CER), (2) protein-rich biopolymers bound to Fe cations and extractable by sulfide, and (3) biopolymers bound to Al cations, extractable with bases (Novak *et al.*, 2003; Park and Novak, 2007; Park *et al.*, 2008; Park and Novak, 2009). In more complex systems, e.g. the activated sludge floc, EPS originate from (i) microbial metabolism or lysis of microorganisms as described above and (ii) from wastewater components accumulated to the floc matrix by sorption processes (Urbain *et al.,* 1993). In addition, hydrolysis processes of macromolecules due to the activity of extracellular enzymes, influence EPS composition (Frølund *et al.,* 1995, Confer and Bruce 1998). Because it is not possible to distinguish between microbially produced EPS, adsorbed material, and hydrolysis products, in this work all three fractions are defined as EPS.

EPS in activated sludges and biofilms are also known to promote cell-cell recognition/communication and protect cells against harmful environmental conditions such as turbulence, dehydration, antibiotics and biocides (Wingender *et al.,* 1999). Furthermore the terms "bound EPS" and "soluble EPS" are used for some biofilm systems (Hsieh *et al.,* 1994; Nielsen *et al.,* 1997). Bound EPS include sheaths, capsular polymers and cell-attached organic material. Soluble macromolecules, colloids, and slimes represent soluble EPS. This means that all polymers outside the cell wall, which are not directly bound to the outer membrane/murein-protein-layer, will be considered extracellular EPS material.

 The main organic fractions detected in activated sludge EPS were proteins, carbohydrates, uronic acids, humic substances, lipids, and fatty acids (Goodwin and Forster, 1985; Urbain *et al.,* 1993; Frølund *et al.,* 1996, Bura *et al.,* 1998; Dignac *et al.,* 1998; Conrad *et al.,* 2003; Wilen *et al.,* 2003). Significant amounts of DNA and RNA were also found (Frølund *et al.,* 1996; Palmgren and Nielsen, 1996). Park and Novak found that each cation-bound fraction of EPS produced a unique SDS-PAGE protein fingerprint, suggesting a different protein composition and therefore accounting for different characteristics conveyed by each fraction (Park *et al.,* 2008). As the pool of EPS proteins is augmented by incoming proteins from

influent stream, by proteins originating from sludge cell lysis, and by proteins actively secreted by sludge microorganisms (Park *et al.*, 2008), the actual role of EPS proteins is most probably very significant, but also very complex. Earlier studies often indicated that polysaccharides were the most abundant and important EPS compound (Brown and Lester, 1980; Horan and Eccles, 1999) but a number of recent studies have shown that the quantity of proteins is about two to three folds higher than polysaccharides in activated sludge EPS (Urbain *et al.,* 1992; Frølund *et al.,* 1996; Nielsen *et al.,* 1996; Higgins and Novak, 1997a; Dignac *et al.,* 1998; Wingender *et al.,* 1999; Liu and Fang, 2002; Comte *et al.,* 2007). It was also reported that glycoproteins are very likely present in activated sludge EPS so that part of the protein and carbohydrate content in EPS arises from the extraction of glycoproteins (Goodwin and Forster, 1985; Jorand *et al.,* 1998; Horan and Eccles, 1999). The general characteristic of bacterial glycoprotein is interesting to note since it often exhibits both acidic characteristic (low isoelectric point) and hydrophobic characteristic (Jorand *et al.,* 1998). Consequently, it can be involved in bacterial aggregation by both electrostatic bond (cation bridging) and hydrophobic interaction.

EPS can be composed of a variety of biopolymers transported to the extracellular milieu by active secretion or export, lysed cellular components from the rupture of cell structure, hydrolyzed or digested exocellular substances, and materials adsorbed from the environment such as in wastewater being fed to an activated sludge system (Urbain *et al.,* 1992; Dignac *et al.,* 1998; Nielsen and Keiding, 1998; Wingender *et al.,* 1999). However, it is mainly unknown how these different-origin EPS are distributed within the floc and contribute to the physiological property of activated sludge flocs. Furthermore, due to the scarcity of molecular investigation on activated sludge EPS, their identity, function, and fate in various stages of the activated sludge system remains veiled. Additional possible functions of EPS are summarized by (Wingender *et al.,* 1999). EPS might act as protective barriers against toxic substances, e.g. heavy metals or certain biocides (disinfectants and antibiotics), predation and dramatic environmental fluctuations (pH, salt content, hydraulic pressure). Furthermore the localization of extracellular enzymes mentioned above, which perform the degradation of exogenous macromolecules and particulate material is well described in the literature. This observation indicates two further functional aspects, which are described in

the following chapters, the involvement of EPS in accumulation and subsequent utilization of these accumulated substances as carbon sources.

(Kim *et al.,* 1998) reported that addition of powder activated carbon (PAC) to the MBR could increase flux permeability by reducing dissolved EPS levels from 121-196 to 91-127 mg/g VSS. (Thuy, 2003) investigated the performance of biological activated carbon (BAC) by adding granular activated carbon into MBR (BAC-MBR) and AS-MBR (activated sludge MBR) to treat inhibitory phenolic compounds. The comparison of the two systems in terms of membrane fouling was carried out. It was found that the TMP suddenly increased in the AS-MBR while the BAC-MBR was linearly increased. TMP in the BAC-MBR after 90 days were slightly higher than that in the AS-MBR, and the bound EPS of the BAC-MBR was higher than that of the AS-MBR. The protein/carbohydrate (P/C) ratio in soluble EPS was high in BAC-MBR (0.86-2.13), but soluble EPS production (0.49-2.03 mgC/gVSS) was low. The P/C ratio and soluble EPS were the two important factors in biofouling.

(Likewise, 1996; Nagaoka *et al.,* 1996) reported that EPS could accumulate in the aeration tank of the membrane separation for activated sludge process, which caused an increase in mixed liquor viscosity and thus in the filtration resistance. (Change and Lee, 1998) noted that the EPS contents of activated sludge could be an indicator for estimating the membrane fouling.

(Mukai *et al*., 2000) estimated flux decline of ultrafiltration membrane at different cultural growth phases i.e. different EPS and metabolic concentrations in AS process. The authors reported that the flux decline was affected by protein to sugar ratio of EPS and metabolic products. Lower permeate flux occurred at higher retention of protein and greater amounts of retained protein during the filtration.

3.2.1. Extraction of EPS of activated sludge

Controversies in EPS extraction studies are also associated with the impact of extracted EPS on sludge characteristics. The quantity of EPS extracted by the cation exchange resin CER procedure was negatively correlated to settling properties (Liao *et al.,* 2001; Wilén *et al.,* 2003a), but related to better dewatering characteristics of activated sludge (Jin *et al.,* 2003;

Mikkelsen and Keiding, 2002). However, EDTA-EPS and glutaraldehyde-EPS reported by (Erikkson and Alm, 1991; Sponza , 2002), respectively, showed negative correlations with both settling and dewatering properties of sludge. Results from the thermal treatment of sludge tended to show either no relationship (Shin *et al.,* 2001) or positive relationship (Goodwin and Forster, 1985) between the amount of extracted EPS and settleablilty of sludge but accounted for poorer dewater ability of sludge (Kang *et al.,* 1989). Despite this confusing information from earlier studies, several important things can be noted. First, as (Novak and Haugan, 1981) suggested two decades ago that there is no universal method for providing quantitative extraction of exocellular biopolymers from sludge floc. Considerable disagreement regarding extraction efficiency between different methods and the low extractability of EPS, even from the best method designated in each study (typically, less than 100 mg EPS/g solids), supports this statement. Second, it is unlikely that the EPS extracted by a single method is representative of EPS in sludge floc.

Controversies about the impact of EPS on sludge characteristics have often been attributed to the different extraction methods with different experimental approaches (cultures, extraction time, shearing force, etc). However, the varying composition of EPS such as the quantity and ratio of proteins and polysaccharides associated with different extraction methods indicate that EPS extracted by different treatments could be qualitatively different and this is more likely the reason for the differences reported. Furthermore, it was seen from the reviewed literature that some types of EPS are highly selective for certain kind of cations over others. Since several extraction methods are specific for certain cations in floc, the extracted materials by different treatments should also be different.

3.2.2. Effect of hospital wastewaters on extracellular polymeric substances formation in municipal wastewater

Previous studies have identified the extracellular polymeric substances (EPS) or soluble microbial products (SMP) as one of the most significant factors responsible for membrane fouling (Drews *et al.,* 2006; Janga *et al.,* 2007; Judd, 2008; Le-Clech *et al.,* 2006; Meng *et al.,* 2009). (Delgado, 2009) confirmed that presence cyclophosphamide presence induced a modification of biological suspended solids. The modifications in the biomass and in the bulk solution appeared to influence the membrane performance.

(Avella *et al.,* 2009) studied the effect of the cyclophosphamide and its mean metabolites on extracellular polymeric substances (EPS) formation and this study confirmed that cyclophosphamide and its mean metabolites in the studied concentrations range influenced the biomass exopolymer production. Clearly that cyclophosphamide presence induced an increase in soluble EPS. The increase of these macromolecular species may be attributed to a protection mechanism. (Laspidou and Rittmann, 2002; Aquino and Stuckey, 2004) observed an increased concen- tration of soluble EPS with a high molecular size in anaerobic chemostat in the presence of toxicants (chloroform or chromium). (Henriques and Love, 2007) found that the EPS matrix inside sludge flocs was a protective barrier for bacteria exposed to chemicals toxins such the octane and cadmium.

(Aquino and Stuckey, 2004) study on soluble microbial products (SMP) in bioreactor spiked with chloroform or chromium: they observed enhanced soluble microbial production, composed mainly of PR and PS and no change in SMP composition in toxic's presence. They suggested that some SMP might be deliberately excreted by micro-organisms in cell to cell communication (quorum sensing). It is now established that the quorum sensing influences the biofilm development or aggregates dispersion (Parsek and Greenberg, 2005) regulating the excretion of PS or PR for biomass survival. It was found that bacteria are a thousand times more resistant to antibiotics in a biofilm than in liquid suspension (Everst, 2006).

3.3. Physic parameters of activated sludge

Floc formation and settling in activated sludge can be assessed using two measurements, namely the MLSS and SVI. MLSS and SVI are routine tests at the macro scale to assess performance of the reactor. MLSS is a measure of suspended solids in the sample. Although flocculation is not greatly affected by the concentration of suspended solids, there are reports in the literature describing the negative influence of high MLSS on effluent quality (Chapman, 1983). MLSS is a measure of mixed liquor suspended solids and this measure includes the total weight of microorganisms, EPS, organic waste, suspended waste and any other particulate in wastewater (Goddard, 1987).

SVI is a measure of sludge settleability. SVI is defined as the volume in millimeters occupied by one gram of suspension after 30 min. It indirectly measures morphology of flocs, and is a

physical characteristic of activated sludge (Liao *et al.,* 2006; Schmid *et al.,* 2003). SVI is measured at the macro level and it tracks the settling of a sludge sample rather than the settling of one single particle. SVI needs to be measured as a function of MLSS. The MLSS consideration is only accurate for sludge samples up to 4000 mg/L, MLSS values higher than 4000 mg/L would introduce errors in the SVI measurement (Dick and Vesilind, 1969). This makes SVI theoretically not supported, but it is a useful assessment of process control. Furthermore, since it is simple, inexpensive, and fast this test is still considered to be a routine test (Dick and Vesilind, 1969; Finch, 1950).

SRT is not a test but an operational parameter that states, how long the sludge has been retained; in other words, it is the cell residence time in a reactor. SRT may influence many other characteristics of activated sludge, including: hydrophobicity, surface charge, surface irregularity and EPS, (Liao *et al.,* 2001; Liao *et al.,* 2006). In addition to SRT, other carefully controlled operational parameters are essential to microbial well-being. Microbial cells could be considered an ongoing progress of evolution and as a result, they demand certain optimized conditions for their survival. These conditions include: pH, temperature, food to microorganism ratio and ratio of different nutrients (Abbassi *et al.,* 2000; Barr *et al.,* 1996; Jenkins *et al.,* 2003; Liu *et al.,* 2002). The above conditions are all necessary for the survival of microorganisms in their niche. In WWTP, the above conditions are not easy to maintain optimally at all times due to parameters such as variability of influent water or weather conditions. When the above conditions are not optimized, the microbial community may change (Boon *et al.,* 2002). Changes in the community may cause inefficiencies in reactor performance along with changes in settleability and/or formation of solid/liquid interfaces (Bruus *et al.,* 1992; Jin *et al.,* 2004; Nielsen *et al.,* 1996). Formation of solid/liquid interfaces is dependent on the stabilization of physicochemical properties in a floc (Lee *et al.,* 1997; Liao *et al.,* 2001; Liu *et al.,* 2009).

4. Analyses instruments

4.1. Activated sludge morphology

Activated sludge is a complex mixture of flocs, smaller cell aggregates, and both organic and inorganic particles suspended in water. The activated sludge floc is a complicated structure composed of biotic and abiotic components. The general structure of a floc is a result of the selective pressure in the wastewater treatment plant, favoring dense aggregates with good settling properties. The biotic community of an activated sludge floc is composed of both prokaryotes – *Bacteria* and some *Archaea* – and eukaryotes – protozoan and often metazoan organisms (Eikelboom, 2000). The actual community composition is dynamic and is a net result of the influent wastewater composition and the conditions inside the treatment plant.

A typical activated sludge floc composed of bacterial cells growing in dense, grape-shaped microcolonies, as filaments or as single cells embedded in the matrix of extracellular polymeric substances (EPS) or attached to filamentous organisms (Jorand *et al*., 1995; Snidaro *et al*., 1997; Jenkins *et al*., 2003). Filamentous bacteria are generally recognized as 'backbones' of a floc, responsible for its mechanical strength, as well as settling properties (Ekama *et al*., 1997). The EPS matrix, composed of several fractions, is dense and sticky, glue-like material, responsible to a large degree for floc and microcolony integrity. In the EPS matrix many holes, cavities and channels are present, which make up for the large surface area of flocs and facilitate water and nutrient transport to the cells growing deep in the floc structure (Liss *et al*., 1996; Daims *et al*., 2001; Chu and Lee, 2004). The EPS matrix can be regarded as a typical gel because of its swelling/deswelling properties and divalent cation bridging (Keiding *et al*., 2001). This is extremely important for the floc properties, which determine the behavior of activated sludge in full-scale processes like settling, dewatering and gravity drainage.

Several studies have shown that the settling and the compaction properties of the activated sludge are directly related to the flocs structure, which depends on a group of chemical, physical and biological factors that significantly influence the balance between filamentous and floc-forming bacteria (Pujols and Canler , 1992), leading to changes in the structure and, thus, in the morphological properties of microbial aggregates. In this way, it is possible to

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establish relationships between sludge settling indexes and several parameters that characterize the morphology of microbial flocs (Námer and Ganczarczyk, 1993; Li and Ganczarczyk, 1987, 1988, 1990, 1992), being these relationships useful for monitoring the settling stage in activated sludge systems. The size of activated sludge flocs is typically 40 to 125 μm, but values down to 25 μm and up to 1000 μm have been reported (Frølund *et al.*, 1996; Ekama *et al.*, 1997; Eikelboom, 2000; Jenkins *et al.*, 2003). The floc size is a net result of the floc strength and the mechanical stresses that the floc is subjected to, whereas the floc strength results from a range of chemical and biological factors. All in all, the floc size is a very dynamic floc characteristic with many implications on sludge macroscopic properties and sludge behavior in large-scale processes. The effective dewatering of activated sludge by gravity drainage depends on a number of physicochemical and microbiological factors, the most important of which seems to be the particle size distribution, similarly to the case of dead-end filtration of abiotic suspensions. Deflocculation, a process of floc disruption into smaller fragments, is especially damaging to the drainage process. Small floc fragments can easily penetrate the cake voids and close the pores (process known as blinding), which leads to increased drag, slower drainage and progressing cake compression.

The presence of small particles in the activated sludge suspension has been shown to decrease dewater ability many times (Karr and Keinath, 1978; Barber and Veenstra, 1986; Mikkelsen *et al.*, 1996). Since deflocculation of activated sludge flocs is a direct result of reduced floc strength (Mikkelsen and Keiding, 1999), the knowledge of floc strength and factors affecting it can be effectively used to investigate the phenomena behind the quality of sludge in terms of gravity drainage. Floc strength can be regarded as a sum of all the interactions that bind bacteria and floc constituents together. The four most commonly cited floc-binding interactions are the DLVO-type interactions (Hermansson, 1999), bridging of EPS with divalent (Eriksson and Alm., 1991) and trivalent cations (Nielsen and Keiding, 1998), hydrophobic interactions (Urbain *et al.*, 1993), and physical entanglement of floc entities (Rijnaarts *et al.*, 1995). All these interactions can be affected by both physico-chemical properties of bulk liquid and biological activity of bacteria inhabiting the flocs, which makes the floc strength a continuously changing parameter, the magnitude of which can be managed with a number of strategies. According to the DLVO theory, bacterial adhesion to floc surface can be increased by increasing the ionic strength of the solution. This effect is

expected to result from decreasing the double layer thickness and decreasing the surface potential, which would eventually act against the electrostatic repulsive forces (Hermansson, 1999).

4.2. Microbial composition and activity

Behind the macroscopic physico-chemical properties of activated sludge flocs, and the EPS matrix composition and function, stand the sludge microorganisms. Even though bacterial cells only make up from 10-20% of the total sludge organic matter (Nielsen and Nielsen, 2002), the composition of sludge microbiota determines the amount and composition of EPS and therefore influences the overall floc characteristics. It has been shown that different groups of bacteria influence the floc strength to a different extent, i.e. that *Beta-*, *Gamma-*, and *Deltaproteobacteria* form relatively strong microcolonies, while colonies of other bacteria like *Alphaproteobacteria* and *Firmicutes* are rather weak (Klausen *et al.*, 2004). This claim is supported by the findings that sludge supernatant and the settled floc differ in microbial composition (Morgan- Sagastume *et al.*, 2008) and that sludge flocs generally have loosely and strongly attached fractions of cells and EPS (Keiding and Nielsen, 1997; Liao *et al.*, 2002; Sheng *et al.*, 2006). The easily detachable fraction of approximately 5-15% of cells can be removed from flocs by shear forces alone, the strongly attached fraction of further 15-40% of cells requires certain physico-chemical treatments in addition to shear forces in order to deflocculate, and the remaining 50-75% of cells cannot be removed from flocs (Larsen *et al.*, 2008). Therefore, it becomes clear that the bacterial community composition determines how a given sludge reacts to a given set of factors and therefore how a given treatment influences floc strength, floc size distribution and, as a consequence, sludge dewater ability and draining characteristics (Klausen *et al.*, 2004). A good balance between filamentous and flocforming bacteria favor the formation of large, dense and strong flocs desirable for adequate settling and compaction of the activated sludge. Misbalance could induce filamentous bulking caused by an overgrowth of filamentous bacteria or disperse growth (pin point floc) provoked by a scarce growth of floc-forming bacteria. The filamentous bulking promotes the formation of highly irregular flocs causing a decrease of settling speed as well as low sludge compaction, while the disperse growth leads to the formation of small and lights flocs that not settle, resulting in a very turbid effluent with high concentration of suspended matter. (Jenkins *et al.*, 1993).

Several techniques have been proposed in literature in order to describe the complex structure of the flocs in terms of the material organization within the aggregates. These techniques have allowed to known the physical aspect of the floc (filament size and fractal dimension), the granulometric distribution of the floc sizes (measured by photographic technique in free settling, Coulter Counter, laser diffraction and Malvern counter, etc) and the consequences of bio-flocculation on flow properties (rheological measurements and settling rates).

4.3. Microscopic techniques

4.3.1. Image Analysis procedure

Various methods have been established to measure the size of activated sludge flocs. The most commonly used approach is microscopy (Barbusinski and Koscielniak, 1995). It represents an excellent technique for directly examining the flocs. However, for manual microscopy, elaborate sample preparation is necessary and only a few particles can be examined. More recently, by connecting the microscope to automated image analysis software, a faster evaluation of activated sludge floc properties became possible (Grijspeerdt and Verstraete, 1997). Another technique used for characterising the activated sludge floc size and size distribution is the Coulter Counter (Andreadakis, 1993). This technique requires sample suspension in an electrolyte, which can create structural disturbance on biological flocs or might cause clogging of the aperture during the measurement of the large size particles.

The recent development of image analysis technique has enabled a more complete understanding of the aggregates physical structure and morphology. Image analysis has become a fundamental tool with great applications within the Environmental Science. In aerobic activated sludge systems, it has been applied for morphological characterization of microbial flocs, allowing the estimation of different parameters of the Euclidian geometry (Grisjpeerdt and Verstraete, 1996, 1997; Jin *et al.*, 2003; Amaral, 2003), the fractal analysis of contour of these aggregates and other aspects such as detection and counting of filaments (Li and Gaczarczyk, 1989; da Motta *et al.*, 2001). These morphological parameters have been correlated with settling properties of activated sludge, estimated as Sludge

Volume Index (SVI) (Grijspeerdt and Verstratete, 1997; da Motta *et al*., 2001; Amaral, 2003), in order to monitor filamentous bulking in wastewater treatment plants. The floc size and size distribution have been often reported in literature as outcomes of a particular measurement technique and less importance has been given to the influence of the measurement technique on the results. Since operation of various devices is based on a broad range of measurement principles, it is expected that different results are obtained.

Moreover, for the case of activated sludge, due to the biological fragile and irregular structure of the flocs, the results may often lead to a misinterpretation of the data.

Experimental setup of the system to the digital image recording

For the aspired automatic regulation of the biological stages of wastewater treatment plants, development and implementation of procedures are necessary that start with taking digital photographs of activated sludge samples by means of a microscope and a CCD camera. The following automatic image processing by algorithms of the digital image processing and the final statistical analysis enable a correlation of data determined the in this way with the operating conditions of the wastewater treatment plant. Images of activated sludge samples are detected with the help of the CCD camera attached at the microscope, screened into a pixel picture and read as an analogue video signal into the Frame Grabber (figure 15). The Frame Grabber changes the video signal with an 8-bit-A/D-transducer into 256 grey tones.
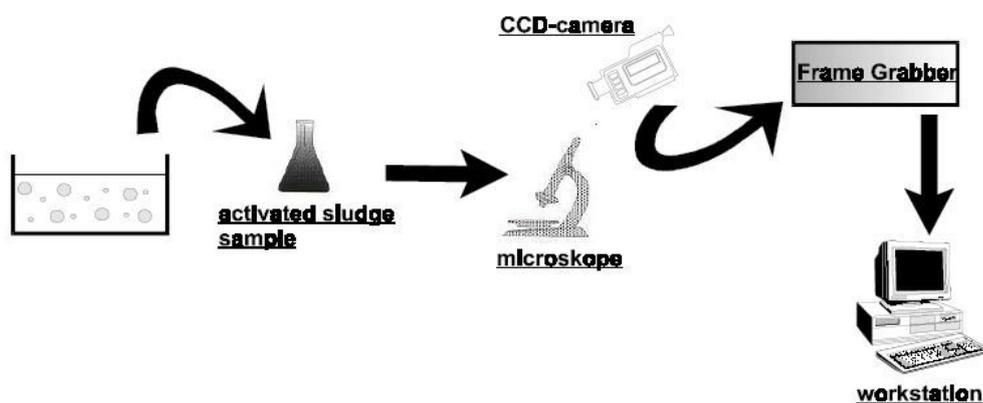
Figure 15: Experimental setup of the system for digital image recording

The available digital image of the activated sludge sample is stored as a file and can be processed subsequently with algorithms of digital image processing. The principle aim of the image processing process is the extraction of certain information from the digital images, so that a scene or individual objects from this images and their relation in the scene can be interpreted and will be learned by a machine. The first step of the digital image processing is the improvement of the quality of the microscopic digital images by image processing measures. Some of these measures are for example the histogram balance or the median filtering. The recognition of an object in a scene is only possible if it is different from other objects and from the background of the scene. A subtask of the image processing exists thus in the division of a picture into meaningful fields and regions, which are different. This process is called segmentation.

The segmentation of the original microscopic digital image (figure 16-A) can be made by using an edge detection algorithm. Objects are separated from their background defining the edges of the object as the modification of the light intensity, this object in the picture contents. A two-dimensional light intensity modification can be described with the help of a function. The turning point of this function represents a point of edge, because at this point the light intensity changes fastest. The aim of edge the detection exists in the determination of such points of edges. The determination of the points of edge is achieved by a calculation of local extrema. The actual point of edge is selected, after a check of the local maximums in different directions. The result of edge detection is the gradient image (figure 16-B), which has to be binarised in the following step.

A   B
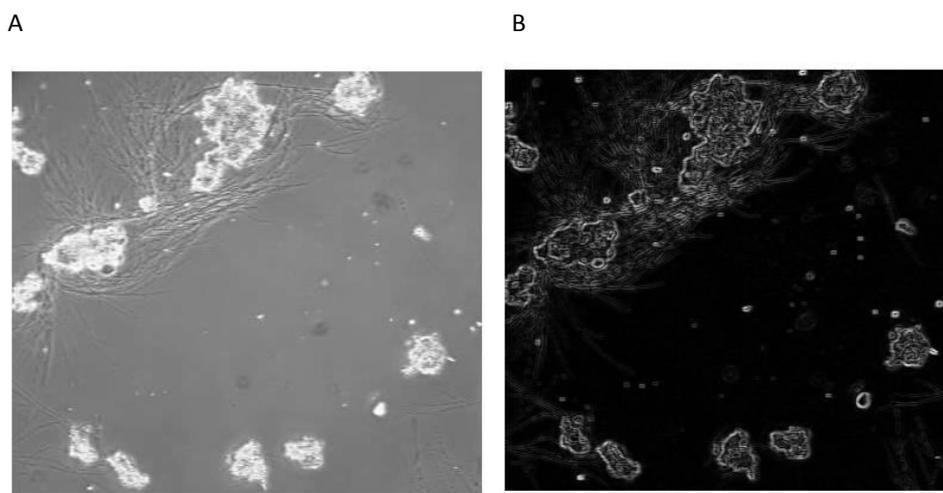
Figure 16: (a) Original and (b) gradient microscopic images of a bulking sludge

4.3.2. Confocal Laser Scanning Microscopy

The technique of laser scanning and spinning disk confocal fluorescence microscopy has become an essential tool in biology and the biomedical sciences, s well as in materials science due to attributes that are to readily available using other contrast modes with traditional optical microscopy (Pawley, 1995 and Masters, 1996). The application f a wide array of new synthetic and naturally occurring fluorochromes has made it possible to identify cells and ub-microscopic cellular components with high degree of specificity amid non-fluorescing material (Mason, 1999). In fact, his confocal microscope is often capable of revealing him presence of a single molecule (Peterman et al., 2004). Through the se of multiply-labeled specimens, different probes a simultaneously identify several target molecules simultaneously, both in fixed specimens and living ells and tissues (Goldman and sector 2005). Although both conventional and confocal microscopes cannot provide spatial resolution below the diffraction limit of specific specimen features, hem detection of fluorescing molecules below such limits s readily achieved.

4.3.2.1. Principles of Confocal Microscopy

The confocal principle in epifluorescence laser scanning microscope is diagrammatically presented in Figure 17. Coherent light emitted by the laser system (excitation source) passes through a pinhole aperture that is situated in a conjugate plane (confocal) with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector (a photomultiplier tube). As the laser is reflected by a dichromatic mirror plane, secondary fluorescence emitted from points on the specimen (in the same focal plane) pass back through the dichromatic mirror and are focused as a confocal point at the detector pinhole aperture.
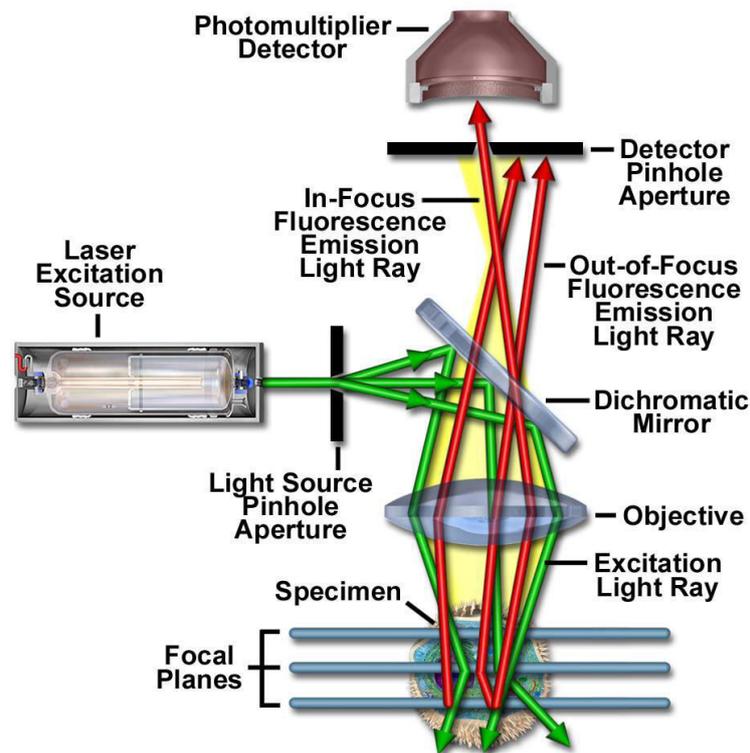
Figure 17: Schematic diagram of the optical pathway and principal components in a laser canning confocal microscope.

The significant amount of fluorescence emission that occurs at points above and below the objective focal plane is not confocal with the pinhole (termed **Outof- Focus Light Rays** in Figure 16) and forms extended Airy disks in the aperture plane (Stelzer *et al.,* 2000). Because only a small fraction of the out-of-focus fluorescence emission is delivered through the pinhole aperture, most of this extraneous light is not detected by the photomultiplier and does not contribute to the resulting image. The dichromatic mirror, barrier filter, and excitation filter perform similar functions to identical components in a wide field epi-fluorescence microscope (Rost *et al.,* 1992). Refocusing the objective in a confocal microscope shifts the excitation and emission points on a specimen to a new plane that becomes confocal with the pinhole apertures of the light source and detector.

In laser scanning confocal microscopy, the image of an extended specimen is generated by scanning the focused beam across a defined area in a raster pattern controlled by two high-speed oscillating mirrors driven with galvanometer motors. One of the mirrors moves the beam from left to right along the **x** lateral axis, while the other translates the beam in the **y** direction. After each single scan along the **x** axis, the beam is rapidly**.** Wide field versus

confocal microscopy illumination volumes, demonstrating the difference in size between point scanning and wide ield excitation light beams. Claxton, Fellers, and Davidson transported back to the starting point and shifted along the **y** axis to begin a new scan in a process termed **fly back** (Webb, 1995). During the fly back operation, image information is not collected. In this manner, the area of interest on the specimen in a single focal plane is excited by laser illumination from the scanning unit.

4.3.2.2. Advantages and disadvantages of confocal microscopy

The primary advantage of laser scanning confocal microscopy is the ability to serially produce thin (0.5 to 1.5 micrometer) optical sections through fluorescent specimens that have a thickness ranging up to 50 micrometers or more (Sandison and W. Webb; 1994). With most confocal microscopy software packages, optical sections are not restricted to the perpendicular lateral (**x**-**y**) plane, but can also be collected and displayed in transverse planes. Vertical sections in the **x**-**z** and **y**-**z** planes (parallel to the microscope optical axis) can be readily generated by most confocal software programs. Most of the software packages accompanying commercial confocal instruments are capable of generating composite and multi-dimensional views of optical section data acquired from z-series image stacks. The three-dimensional software packages can be employed to create either a single three-dimensional representation of the specimen or a video (movie) sequence compiled from different views of the specimen volume.

In many cases, a **composite** or **projection view** produced from a series of optical sections provides important information about a three-dimensional specimen than a multi-dimensional view (Conchello *et al.,* 1994-2005).

Advances in confocal microscopy have made possible multi-dimensional views (Conchello *et al.,* 1994-2005) of living cells and tissues that include image information in the x, y, and z dimensions as a function of time and presented in multiple colors (using two or more fluorophores). Additional advantages of scanning confocal microscopy include the ability to adjust magnification electronically by varying the area scanned by the laser without having to change objectives. This feature is termed the **zoom factor**, and is usually employed to

adjust the image spatial resolution by altering the scanning laser sampling period (Pawley, 1995; Centonze, 1995).

Disadvantages of confocal microscopy are limited primarily to the limited number of excitation wavelengths available with common lasers (referred to as **laser lines**), which occur over very narrow bands and are expensive to produce in the ultraviolet region (Gratton, 1995).

Another downside is the harmful nature (Ashkin, 1987) of high-intensity laser irradiation to living cells and tissues, an issue that has recently been addressed by multiphoton and Nipkow disk confocal imaging. Finally, the high cost of purchasing and operating multi-user confocal microscope systems (DeMaggio, 2002), which can range up to an order of magnitude higher than comparable wide field microscopes, often limits their implementation in smaller laboratories.

4.3.2.3. Fluorophores for confocal microscopy

Biological laser scanning confocal microscopy relies heavily on fluorescence as an imaging mode, primarily due to the high degree of sensitivity afforded by the technique coupled with the ability to specifically target structural components and dynamic processes in chemically fixed as well as living cells and tissues. Many fluorescent probes are constructed around synthetic aromatic organic chemicals designed to bind with a biological macromolecule (for example, a protein or nucleic acid) or to localize within a specific structural region, such as the cytoskeleton, mitochondria, Golgi apparatus, endoplasmic reticulum, and nucleus (Haugland *et al.,* 2005) Other probes are employed to monitor dynamic processes and localized environmental variables, including concentrations of inorganic metallic ions, pH, reactive oxygen species, and membrane potential (Lemasters *et al .,* 1999). Fluorescent dyes are also useful in monitoring cellular integrity (live versus dead and apoptosis), endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction, and enzymatic activity (Johnson, 1998) addition, fluorescent probes have been widely applied to genetic mapping and chromosome analysis in the field of molecular genetics.

4.3.2.4. Basic characteristics of fluorophores

Fluorophores are catalogued and described according to their absorption and fluorescence properties, including the spectral profiles, wavelengths of maximum absorbance and emission, and the fluorescence intensity of the emitted light (Johnson, 1998). One of the most useful quantitative parameters for characterizing absorption spectra is the molar extinction coefficient (denoted with the Greek symbol **e**, see Figure 18(a)), which is a direct measure of the ability of a molecule to absorb light. The extinction coefficient is useful for converting units of absorbance into units of molar concentration, and is determined by measuring the absorbance at a reference wavelength (usually the maximum, characteristic of the absorbing species) for a molar concentration in a defined optical path length. The quantum yield of a fluorochrome or fluorophore represents a quantitative measure of fluorescence emission efficiency, and is expressed as the ratio of the number of photons emitted to the number of photons absorbed. In other words, the quantum yield represents the probability that a given excited fluorochrome will produce an emitted (fluorescence) photon. Quantum yields typically range between a value of zero and one, and fluorescent molecules commonly employed as probes in microscopy have quantum yields ranging from very low (0.05 or less) to almost unity. In general, a high quantum yield is desirable in most imaging applications. The quantum yield of a given fluorophore varies, sometimes to large extremes, with environmental factors, such as metallic ion concentration, pH, and solvent polarity (Johnson, 1998)
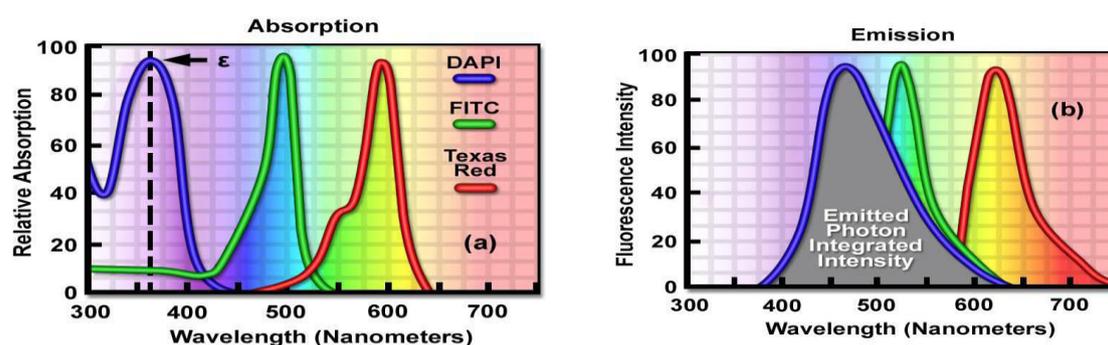
Figure 18: Fluorescent spectral profiles, plotted as normalized absorption or emission as a function of wavelength, for popular synthetic fluorophores emitting in the blue, green, and red regions of the visible spectrum. Each profile is identified with a colored bullet in (a), which illustrates excitation spectra. (b) The emission spectra for the fluorophores according to the legend in (a).

In most cases, the molar extinction coefficient for photon absorption is quantitatively measured and expressed at a specific wavelength, whereas the quantum efficiency is an assessment of the total integrated photon emission over the entire spectral band of the fluorophore (see Figure 18(b)). As opposed to traditional arc-discharge lamps used with the shortest range (10-20 nanometers) band pass interference filters in wide field fluorescence microscopy, the laser systems used for fluorophore excitation in scanning confocal microscopy restrict excitation to specific laser spectral lines that encompass only a few nanometers (Pawley, 1995 ; Hibbs, 2004). The fluorescence emission spectrums for both techniques, however, is controlled by similar band pass or long pass filters that can cover tens to hundreds of nanometers (Hibbs, 2004). Below saturation levels, fluorescence intensity is proportional to the product of the molar extinction coefficient and the quantum yield of the fluorophore, a relationship that can be utilized to judge the effectiveness of emission as a function of excitation wavelength(s).

4.3.2.5. Traditional fluorescent dyes

Many of the classical fluorescent probes that have been successfully utilized for many years in wide field fluorescence (Johnson, 1998; Kasten 1999), including fluorescein isothiocyanate, Lissamine rhodamine, and Texas red, are also useful in confocal microscopy. Fluorescein is one of the most popular fluorochromes ever designed, and has enjoyed extensive application in immunofluorescence labelling. This xanthene dye has an absorption maximum at 495 nanometres, which coincides quite well with the 488 nanometer (blue) spectral line produced by argon ions and krypton-argon lasers, as well as the 436 and 467 principal lines of the mercury and xenon arc-discharge lamps (respectively). In addition, the quantum yield of fluorescein is very high and a significant amount of information has been gathered on the characteristics of this dye with respect to the physical and chemical properties (Wessendorf and Brelje, 1992). On the negative side, the fluorescence emission intensity of fluorescein is heavily influenced by environmental factors (such as pH), and the relatively broad emission spectrum often overlaps with those of other fluorophores in dual and triple labeling experiments (Johnson, 1998; Wessendorf and Brelje, 1992; Entwistle and Noble, 1992).

Tetramethyl rhodamine (**TMR**) and the isothiocyanate derivative (**TRITC**) are frequently employed in multiple labeling investigations in widefield microscopy due to their efficient excitation by the 546 nanometer spectral line from mercury arc-discharge lamps. The fluorochromes, which have significant emission spectral overlap with fluorescein, can be excited very effectively by the 543 nanometer line from helium-neon lasers, but not by the 514 or 568 nanometer lines from argon-ion and krypton-argon lasers (Entwistle and Noble, 1992). When using krypton-based laser systems, Lissamine rhodamine is a far better choice in this fluorochrome class due to the absorption maximum at 575 nanometers and its spectral separation from fluorescein. Also, the fluorescence emission intensity of rhodamine derivatives is not as dependent upon strict environmental conditions as that of fluorescein. Several of the acridine dyes, first isolated in the nineteenth century, are useful as fluorescent probes in confocal microscopy (Wessendorf and Brelje, 1992).

The most widely utilized, acridine orange, consists of the basic acridine nucleus with dimethylamino substituents located at the 3 and 6 positions of the tri-nuclear ring system. In physiological pH ranges, the molecule is protonated at the heterocyclic nitrogen and exists predominantly as a cationic species in solution. Acridine orange binds strongly to DNA by intercalation of the acridine nucleus between successive base pairs, and exhibits green fluorescence with a maximum wavelength of 530 nanometers (Johnson, 1998; Wessendorf and Brelje, 1992; Darzynkiewicz, 1990). The probe also binds strongly to RNA or single stranded DNA, but has a longer wavelength fluorescence maximum (approximately 640 nanometres; red) when bound to these macromolecules. In living cells, acridine orange diffuses across the cell membrane (by virtue of the association constant for protonation) and accumulates in the lysosomes and other acidic vesicles. Similar to most acridines and related polynuclear nitrogen heterocycles, acridine orange has a relatively broad absorption spectrum, which enables the probe to be used with several wavelengths from the argon-ion laser.

Another popular traditional probe that is useful in confocal microscopy is the phenanthridine derivative, propidium iodide, first synthesized as an anti-trypanosomal agent along with the closely related ethidium bromide). Propidium iodide binds to DNA in a manner similar to the acridines (via intercalation) to produce orange-red fluorescence centered at 617 nanometers (Waring, 1965; Arndt-Jovin and Jovin, 1989). The positively charged fluorophore also has a high affinity for double-stranded RNA. Propidium has an absorption maximum at 536

nanometers, and can be excited by the 488-nanometer or 514-nanometer spectral lines of an argon-ion (or krypton-argon) laser, or the 543-nanometer line from a green helium-neon laser. The dye is often employed as a counterstain to highlight cell nuclei during double or triple labeling of multiple intracellular structures. Environmental factors can affect the fluorescence spectrum of propidium, especially when the dye is used with mounting media containing glycerol. The structurally similar ethidium bromide, which also binds to DNA by intercalation (Waring, 1965), produces more background staining and is therefore not as effective as propidium.

4.3.2.6. EPS analysis with confocal laser scanning microscopy and chemical analysis

CLSM was used for the identification of bacteria and EPS distribution within the biofilm matrix. Based on the work of (Staudt *et al.,* 2003) EPS glyco conjugates were stained with the Aleuria aurantia lectin (LINARIS Biologische Produkte GmbH, Wertheim-Bettingen, and Germany) labeled with AlexaFluor_ 488 (invitrogen/Molecular Probes, Eugene, USA). For the identification of bacteria the nucleic acid stain SYTO60 (invitrogen/ Molecular Probes, Eugene, USA) was applied following the Probes, Eugene, USA) was used to stain proteins within the biofilm matrix (Lawrence et *al.,* 2003). Image stacks were created with a Zeiss LSM510 META confocal laser scanning microscope (Carl Zeiss Micro Imaging GmbH, Jena, Germany), controlled by means of the AIM software (version 3.2, Carl Zeiss Micro Imaging GmbH, Jena, Germany). The Zeiss LSM510 META is equipped with different lasers, offering several excitation wavelengths. For fluorescence excitation two wavelengths were used: 488 and 633 nm. For the direct observation of biofilms on slides a water immiscible lens (40x magnification, N.A. ¼ 0.8) was used. For each measurement, one of the marked areas on the slide was chosen randomly. On this spot, five points were scanned to determine the distribution of EPS and nucleic acids. The pinhole for each scanned channel was adjusted to scan all channels with an identical optical slice thickness (North, 2006) of 0.78 mm.

In addition to lectin binding analysis with CLSM, a chemical analysis of the EPS was performed using a DOWEX cation exchange resin to break down the network of the EPS by exchange of divalent cations (Ca^{2+}, Mg^{2+}) as described in literature (Comte *et al.,* 2006; Frolund *et al.,* 1996; Jahn and Nielsen, 1995; Lowry *et al.,* 1951; Nielsen and Jahn, 1999). Thereby 70 g DOWEX cation exchange resin (Type Na) were added per 1 g of dried organic

matter of biofilm sample. Determination of dried organic matter was performed before following the rules of DIN EN 12880 (Deutsches Institut fur Normung e.V, 2000). Reaction has been carried out with approximately 10 g of rinsed biofilm sample in a shaking flask for 1.5 h under stirring (900 rpm) at 4 C°. Used cation exchange resin was separated by centrifugation at 4300 g for 5 min. Resulting supernatant was centrifuged again at 4300 g for 15 min (2 times in cooled environment) and filtered through a cellulose acetate filter with pore diameter of 0.45 mm. In this supernatant proteins and humic substances were determined by the methods described by Lowry *et al.,* 1951) and Frolund *et al.,* 1995), respectively. The concentration of carbohydrates in the supernatant was determined by the anthrone method (Raunkjaer *et al.,* 1994).

4.3.2.7. Digital image analysis

Image analysis was performed with the freely available software ImageJ version 1.39i (http://rsb.info.nih.gov/ij/index. html) including the LSM-Reader plugin to open LSM5 formatted image stacks created by the microscope software. The tool J Image Analyzer 1.1, which is based on the performance of Image J and handles LSM5 formatted image stacks, was programmed for quantitative analysis. By setting a threshold, pixels with intensity below the threshold were assigned to the background. All other pixels were set to the foreground. Due to the individual image adjustment during the image stack acquisition, the threshold was chosen manually for each image stack. It has to be stressed that the pitfalls of threshold setting by the operator is well known (Staudt *et al.,* 2004; Yang *et al.,* 2001). Thus, the conditions during digital image analysis were kept constant for each image analysis. The same monitor was used including settings for brightness and contrast. To avoid hardware-based influences, all DIA were performed by the same person to avoid individual influences. Nevertheless, operating manually allows optimization of the images with respect to structural information.

The foreground pixels were counted and the coverage C of every single image of the image stack was quantified. Additionally, an average coverage C*stack* was calculated for each image stack. (Eq. 4)

$$\frac{1}{} \quad n = n_{max}$$

$$C_{stack} = \frac{1}{n_{max}} \sum_{n=1} C$$

With n = number of slices   (Eq. 4)

$$C = \frac{1}{n_{max}} \sum_{n=1}^{n=n_{max}} C_{stack}$$

With n = number of image stacks.

In a next step, all values of average coverage C stack of all analyzed image stacks of one slide taken from a funnel with a Reynolds number of 1000, 2500 and 4000, respectively, were averaged once more to obtain one single value C representing the averaged amount of scanned EPS glyco conjugates and nucleic acids on one slide. To better visualize the amount of EPS glyco conjugates and nucleic acids detected, stacks of 50 single images (slices) were evaluated as packages. The sum of coverage within such packages of 50 single images (slices) was calculated by (Eq. 5) (Wagner et al. 2008).

$$SC_{stack} = \sum_{slices\ n}^{slices\ n\ +49} C$$ (Eq. 5)

With n = 1, 51, 101…

$$SC = \frac{1}{n_{max}} \sum_{n=1}^{n=n_{max}} (SC_{stack} / 50)$$

With n = number of image stacks.

4.3.3. Fluorescence spectroscopy

Fluorescence is a specific type of photoluminescence, the general term used to describe the interaction that occurs when molecules are excited by the absorption of photons of electromagnetic radiation and then, consequently, the re-emission of light energy. The

phenomenon of fluorescence occurs when a beam of light is passed through a sample and the photons of light excite the electrons of the molecules in the sample. The electrons jump into higher energy molecular orbitals and then as they fall back into their original orbitals they emit energy in the form of light. Fluorescence is characterized by this almost immediate re-emission of energy after absorption, the entire event occurring in only 10-12 to 10-9 second (Vogel, 1989).

Fluorescence can be measured through the use of a fluorescence spectrometer. A typical instrument consists of a radiation source, a primary monochromator, a secondary monochromator, a detector, an amplifier, and a readout device. Light from the source of radiation is passed through the primary monochromator, which allows only the wavelength of light required for excitation of the molecules in the sample to pass through. The second monochromator, located at a 90° angle from the incident optical path, absorbs this primary radiant energy, transmitting only the fluorescent radiant energy. The geometrical arrangement of this device makes it particularly sensitive, around three to four orders of magnitude more sensitive than the spectrophotometer, and therefore a very important analytical tool (Dekker and Guilbault, 1990).

Three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy is a rapid, selective and sensitive technique. The outstanding advantage of EEM fluorescence spectroscopy is that information regarding the fluorescence characteristics can be entirely acquired by changing excitation wavelength and emission wavelength simultaneously. Thus, because of its high sensitivity, good selectivity, and non-destruction of samples, EEM fluorescence spectroscopy could be useful for studying the chemical and physical properties of EPS. It can be used to distinguish the fluorescence compounds present in the complex EPS mixtures from various origins. EEM fluorescence spectroscopy has been successfully used to evaluate the characteristics of natural dissolved organic matter and humic substances from various origins (Coble, 1996; Baker 2001; Lu and Jaffe, 2001; Reynolds, 2002; Chen *et al.,* 2003). It has been proven to be a useful technique to differentiate the changes and transformations of organic matter in natural environments. In biological and biochemical fields of study, the fluorescence spectrometer is often used to detect fluorescent probes. There are three classes into which fluorescent probes can be divided: intrinsic probes, extrinsic covalently bonded probes, and extrinsic associating probes.

Tryptophan is one of the three aromatic amino acid residues found in proteins which act as intrinsic fluorophores (the other two amino acids being tyrosine and phenylalanine), (Valeur, 2002) and although typical proteins are comprised of only 1.1 molar percent tryptophan residues, this particular amino acid is a very valuable probe of protein structure (Pokalsky *et al.,* 1995). In comparison to the absorption maxima (λmax) and extinction coefficient (ε) for both tyrosine (λmax=274.8, ε=1405) and phenylalanine (λmax=257.6, ε=195), tryptophan has a higher wavelength of absorption and a much higher extinction coefficient (λmax=279.0, ε=5579). Both of these factors contribute to the dominance of the tryptophan emission signal, making it the "ultimate energy acceptor in proteins" (Dekker and Guilbault, 1990). For this reason, tryptophan can be used as a fluorescent probe to determine the relative concentrations of protein, and hence of organic materials, contained within different samples of wastewater.

4.3.4. IR spectroscopy

The basic principle of the IR spectroscopy is the excitation of polar bonds of molecules by absorption of light in the infrared region of the electromagnetic spectrum. Absorption is primarily between atoms of hydrogen, carbon, oxygen and nitrogen, the so called light atomic bonds (e.g. C-H, C-O, C=C). It causes molecular vibrations with a life time in the order of 10-9 - 10-6 s after excitation. The frequency or wavelength at which atoms of a molecule are excited and start to vibrate is dependent on the types of vibrating atoms (atomic mass and radius), the bond strength and the structure of the molecules.

These mass and structural dependent vibrations are called normal vibrations. Normal vibrations are developed as discrete vibrations of all atoms of a molecule moving in phase with the same frequency but with different amplitudes (KELLNER *et al.,* 2004; HARRIS, 2007). The fundamental modes of normal vibrations are stretching (stretching and shortening of chemical bonds, symmetric or asymmetric), bending (in-plane movement of atoms changing the angle between bonds), wagging (in-phase, out-of-plane movement of atoms, while other atoms of the molecule are in-plane), rocking (in-phase forth and back swinging of atoms in the symmetry plane of the molecule), and twisting (rocking vibration with twisting of the plane during the movement of the atoms) (TWARDOWSKI and ANZENBACHER ,1994). The high sensitivity to changes in composition and structure of normal vibration facilitates a

fingerprint-type identification of polyatomic molecules. IR radiation only causes vibration in polar bonds in which a change of the dipole moment occurs. Due to this fact, non-polar molecules cannot be identified by IR spectroscopy (Kellner *et al.,* 2004). Compared to normal vibrations, absorption bands of functional groups are independent of structure and composition of the molecules.

This independence occurs if the atoms constituting the functional group are significantly lighter or heavier than the neighbouring atoms or if the bond strength in the functional groups differs from those of the bonds in the vicinity. The absorption of functional groups is called group frequency and significantly developed by functional groups containing H atoms or isolated double and triple bonds. The corresponding wavelength region of group frequencies is situated at wave number positions higher than 1300 cm^{-1} and groups containing heavy atoms are found in the FIR region below 400 cm-1. The wavelength range from 1,300 to 400 cm^{-1} is called the fingerprint region and contains bands of absorbance of special significance for the entire molecule (Kellner *et al.,* 2004). With regard to the aim of this thesis; the analysis of sediments, IR spectroscopy enables the identification of both organic and minerogenic components, whereas spectral regions related to minerogenic components are mostly situated in the fingerprint region due to the missing of functional groups with the exception of hydroxyl group. The basis for quantitative analysis of certain sediment components is the Bouger- Lambert-Beer law (see Eq. 6) which demonstrates the direct proportionality of absorbance *A* to concentration **C**, of the light-absorbing species in the sample. The absorbance is expressed as

$$A = \varepsilon b C \qquad \qquad \text{(Eq. 6)}$$

Where ε is the molar absorptivity (M^{-1}cm-^{1}) and *b* is the path length (cm). Absorbance is dimensionless, but the term "absorbance units" after absorbance can be found in the literature. The concentration is usually given in units of moles per liter (M) (HARRIS, 2007). A correct estimation of concentration of a single compound by integrating of peak areas is difficult due to overlapping of various absorbance bands, especially within mixtures like sediments that contain many different compounds. Other common methods based on the peak height, the maximum absorbance, at a certain frequency are affected by the additive

character of absorbance. Therefore the integration of multivariate techniques has been a major advance in quantitative analysis of IR spectra and is now commonly used for data extraction (Griffiths and De haseth, 2007).

4.3.5. Ion Exchange Chromatography

Natural and artificial zeolites (sodium aluminum silicates) have been used for many years to remove calcium and magnesium ions from water because they include metal ions, which are able to exchange places with the other metal ions. Ion exchangers are now being produced which combine a polymer (a resin which acts as an insoluble inert support) and a functional group, which dictates whether the exchanger is anionic or cationic. Acids are usually used as the functional group in cation exchange resins while amines or quaternary ammonium salts are generally used in anion exchange resins.[8] both types of exchangers can be used for the analyzation of wastewater, the dominant ions being Cl^-, NO_2^-, NO_3^-, PO_4^{-3}, and NH_4^+.

The rate of ion exchange, and hence separation of the ions, is governed by their relative affinities. The metal ions in the sample are in constant competition for binding of the functional groups. Generally at equal concentrations the ion with the highest affinity for the functional group will take the binding site and move the slowest through the column. An ion's affinity is determined by its charge and its size: the greater the charge and the larger the size, the higher the affinity. The total cation or anion content of a sample is also able to be determined simply by using either a cation or an anion exchanger and then titrating the H_+ or OH_-, respectively (Robinson, 2005).

4.4. Dosage the pharmaceuticals compounds in wastewaters

The validation and determination of organics micropollutants content in municipal wastewater samples has been determined by two separate ways, liquid chromatography combined with mass spectrometry (LC/MS) and gas chromatography (GC) combined with mass spectrometry (GC/MS). When GC/MS is used to analyze samples of municipal wastewater, there are different steps as opposed to LC/MS. Samples are taken through SPE similar to LC/MS procedures, except when samples are dried down under a stream of nitrogen they are completely dried down and then derivatized with BSTFA ((N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane), (Mari *et al.,* 2009). Derivatization is the process by which a compound is chemically modified to produce a new

compound that can be analyzed by gas chromatography. The use of derivatization helps increase volatility, detectability, and improves chromatographic behavior (Regis Technologies Inc, 2000). After derivatization, the samples are injected onto a GS/MS instrument with specific protocol parameters (Mari *et al.,* 2009; Mustonen *et al.,* 2005).

Liquid chromatography combined with mass spectrometry is the most widely used method for the determination of organics micropollutants in municipal wastewater samples. Two types of liquid chromatography have been used to evaluate organics micropollutants in municipal wastewater samples: high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) (Nuijs *et al.,* 2011).

High performance liquid chromatography and ultra performance liquid chromatography are similar when used during the analysis of illicit drug content in wastewater samples (Nuijs *et al.,* 2011). HPLC is an extremely powerful tool in analytical chemistry used to separate, identify, and quantitate compounds in a sample that can be dissolved in a liquid. HPLC uses high pressure to push solvents through a packed column. With the use of column particle sizes of 5 μm and pump pressures up to 6000 pounds per square inch (psi), HPLC has been used to separate different constituents of a compound since the 1970's (Wang and He, 2011; http://www.waters.com/waters/nav.htm?cid=10048919, 2012). Ultra performance liquid chromatography is a variant of HPLC. UPLC is a much newer technology that has significant increases in resolution, speed, and sensitivity in liquid chromatography. UPLC uses smaller columns with 1 or 2 millimeter internal diameters packed with smaller particles (1.7 micron) and have the ability to deliver mobile phases at 15,000 (psi) (http://www.waters .com/waters/nav.htm?cid=10048919, 2012). Using high-pressure fluidics and smaller particle size columns, along with the optimization of pump, injector, column, and detector technology, UPLC has improved liquid chromatography (Wang and He, 2011, http://www.waters.com/ waters/nav.htm?cid=10048919, 2012).

There are three major types of chromatography used within liquid chromatography: hydrophilic interaction liquid chromatography (HILIC), reversed-phase liquid chromatography (RPLC), and normal phase liquid chromatography (NPLC). Normal phase chromatography is used to separate compounds based on their polarity. NPLC uses a polar

stationary phase or column, which is most often silica, in combination with a non-polar solvent. Solvents usually include hexane, ethyl acetate, or other mobile phases that have a low polarity (Wang and He, 2011). When NPLC is used, non-polar compounds are eluted off at a faster rate than polar compounds (Snyder *et al.,* 1988). Reversed-phase chromatography involves the separation of molecules based on their hydrophobicity. Columns that are used consist of an alkylsilica-based, non-polar sorbent linked with carbon-18 (C18) that allows separation based on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the sorbent (Walker and Rapley, 2008).

Other columns may be used such as carbon-8 or cyano, both of which have a more immediate polarity. Cyano can be used in both NPLC and RPLC (Wang and He, 2011). Two separate mobile phases are used for the separation of molecules. One mobile phase consists of a mixture between water and an organic solvent. The other mobile phase is an organic solvent, methanol or acetonitrile, used to elute analytes from chromatographic columns. The aqueous phase usually contains ammonium formate or ammonium acetate, and has been acidified with formic or acetic acids. This aids in the ionization of the compounds in the positive ionization mode.

The aqueous phase in the negative ionization mode varies from basic, to neutral, or slightly acidic (Nuijs *et al .,* 2011; Castiglioni *et al.,* 2011; van Juijs *et al.,* 2009 ; Bijlsma *et al.,* 2009 ; Boleda *et al.,* 2007). Hydrophilic interaction liquid chromatography (HILIC) works like normal phase liquid chromatography (3). The stationary phase in HILIC is often more polar than the mobile phase and the analytes typically elute in an order opposite that of RPLC (Wang and He, 2011;   Carlsen, 1997). The phases used in HILIC consist of a polar stationary phase and a highly organic mobile phase, usually methanol or acetonitrile. Water is used as an eluting solvent and resolves polar analytes better than reversed-phased columns. Under these conditions small polar compounds are retained by the stationary phase (Gheorghe *et al.,* 2008).

The ionization of drugs and their various metabolites with LC-MS/MS has been carried out with electrospray ionization (ESI). The majority of illicit drugs, their various metabolites, and pharmaceuticals are best ionized in the positive mode. Cannabinoids show good responses in both the positive and negative mode. ESI has one drawback however; it is susceptible to

matrix effects of analyte ionization signal (Castiglioni *et al.,* 2011). Matrix effects often compromise the analysis of samples by LC-MS/MS. Different approaches have been used to account for matrix effects including: matrix-matched standards calibration, sample dilution, and the use of stable isotopically labeled internal standards (Martinez . Most reported methodologies include isotope-labelled internal standards in order to compensate for losses of desired compounds during SPE and/or matrix effects in wastewater matrices (Castiglioni *et al.,* 2011).

Mass Spectrometry

There are two major types of mass spectrometry that have been incorporated within liquid chromatography for analysis of wastewater effluent samples: single quadrupole MS (Q) and triple quadrupole MS (QqQ) (Ferrer and Thurman, 2003). Single quadrupole mass spectrometry contains a single mass filtering quadrupole. This quadrupole works in a selective mode known as Selected Ion Monitoring (SIM). As a set of voltages are applied to the quadrupole this allows for only one ion of a specific mass-to-charge ratio 21 (m/z) to pass while other ions with different m/z are filtered out. This allows for the detection of a single analyte as it passes through the quadrupole (Schreiber, 2010). Triple quadrupole (QqQ) MS incorporates three different quadrupoles as opposed to a single one (Schreiber, 2010). QqQ works using a mode known as Multiple Reaction Monitoring (MRM) which allows for more selectivity and noise reduction (Schreiber, 2010). The first of the three quadrupoles filters out a specific precursor ion based on m/z. The second quadrupole acts as a collision cell to produce a product ion by the collision of the precursor ion with a neutral gas, like nitrogen. This process is known as Collision Induced Dissociation (DIC) producing a product ion that is sent to the third quadrupole. The third quadrupole acts similar to the first where only product ions with a specific m/z are allowed to pass while all others are filtered out (Schreiber, 2010).

There are multiple advantages to using a triple quadrupole as opposed to a single quadrupole. Triple quadrupoles provide a higher selectivity with less interference resulting in less time consuming method development and faster analysis times. There is also a better signal to noise ratio as compared to the single quadrupole providing lower Limits of

Quantitation (LOQ) and better accuracy and reproducibility at lower concentrations (Schreiber, 2010).

5. Conclusion

Scientists as (Pauwels and Verstraete, 2006) and projects conducted by the laboratory (Pills project, SIPIBEL) have been demonstrated that the hospital effluents present really different qualitative and quantitative characteristics (Altin et al., 2003; kosma et al., 2010; Liu et al., 2010; Verlicchi et al., 2010a) in compared with the urban wastewater. Hospital effluents are considered as hotspots for specific compounds discharge in the environment because the concentrations of these compounds, and thus their effects, are higher than in a urban wastewater, even if the total quantity (g/day) is comparatively lower (It is recognized that hospital effluent represents around 20% of the pharmaceutical load in a urban sewer.

For that, hospital wastewater was studied in this work.

Pharmaceutical micropollutant could be detected in soluble or in solid phase, depending on sorption capability. Pharmaceutical micropollutant could by biologically oxidized depending on their biodegradability. Thus, these compounds could be removed from the effluent by different mechanisms and different processes, which are described in the bibliography. Ternes (1998) monitored 32 pharmaceutical drugs and 5 metabolites in municipal WWTP influent and effluent, and in the receiving surface waters. Ternes found mainly the acidic drugs ubiquitously in surface waters in the nanogram-per-liter range. (Khan and Ongerth, 2004) that 29 (58%) of the pharmaceuticals would be present in the influent at concentrations of greater than or equal to 1 µg/l, and 20 (40%) of the pharmaceuticals would still be present in the wastewater at concentrations greater than or equal to 1 µg/l after secondary treatment. (Snyder *et al.,* 2007) reported that concentrations of caffeine, acetaminophen, sulfameth- oxazole, carbamazepine, and gemfibrozil decreased as the compounds passed through the pilot MBR with removal efficiencies varying between 99.1% (sulfamethoxazole) and 99.9% (acetaminophen). (Radjenovic *et al.,* 2009) found that the removal of acetaminophen from the aqueous phase by the MBR was greater than 99% (similar to the CAS). No elimination of gemfibrozil took place by CAS treatment, whereas 30-40% of this compound was eliminated by the MBR. In the same study, carbamazepine remained untreated by both technologies. Removal efficiencies of sulfamethoxazole were

higher by the MBR technology (81%) than by the conventional activated sludge (75%). (Kimura *et al.,* 2005) investigated the ability of submerged MBR at a municipal WWTP to remove six pharmaceuticals and one herbicide (dichlorprop). (Bouju *et al.,* 2008) shows that MBRs should be more efficient on Persistent organic pollutants (POPs) removal than CAS.

In our work, we oriented our studies towards biological processes as activated sludge, and, to increase the productivity, towards fixed biomass as MBBR.

(Heberer *et al.*, 2002) identified diclofenac as one of the most important pharmaceuticals in the anthropic water cycle, with low µg/L concentrations in both row and treated wastewater (3.0 and 2.5 µg/L at the influent and effluent, respectively). As a result of the incomplete removal during conventional wastewater treatment, these compounds were also found in surface waters in the ng/L to low mg/L range (Ternes *et al.,* 1998). (Kinney *et al.*, 2006) showed that organic wastewater contaminants could be detected in the target biosolids with high frequency and high concentration, which suggests that biosolids can be an important source of organic wastewater contaminants to terrestrial environment. (Xia *et al.,* 2005) indicated that the PPCPs that enter wastewater treatment plants can undergo partial or complete transformation and by-products can be discharged to the environment in the final effluent or through biosolids being applied to land.

Due to this results, our study was oriented on the upgrading of biological treatment technologies by used the membrane bioreactors and their improvements.

Previous studies (Serrano *et al.,* 2010) showed that a GAC addition of 0.5 $g.L^{-1}$ directly into the aeration tank of an activated sludge reactor can be a useful tool to increase the removal of the recalcitrant PPCPs carbamazepine, diazepam and diclofenac., (Ng and Stenstrom, 1987) showed that the use of 0.5- 4 $g.L^{-1}$ of PAC may enhance nitrification rates by 75 and 97%, whereas other authors observed an improvement of organic matter removal as well as a significant decrease of toxicity caused by certain inhibitors on the nitrification process (Widjaja *et al.,* 2004). In fact, activated carbon is a suitable support for bacterial attachment, being possible in this way to enhance the retention of the more slowly growing bacteria, such as nitrifies (Thuy and Visvanathan, 2006; Aktas and Cecen, 2001). The overall results confirm slightly the importance of using the activated carbon to upgrading the treatment systems.

The occurrence of antibiotic in effluent could have two consequences: the modification of the biomass morphology and the promotion of antibiotic resistances. Sulfonamides, fluoroquinolone, and macrolide antibiotics show the highest persistence and are frequently detected in wastewater and surface waters (Huang *et al.,* 2001). Sulfamethoxazole is one of the most detected sulfonamides (Brown *et al.,* 2006; Yang *et al.,* 2005) that was reported with various concentrations and up to ca. 8mg/L (in raw influent in China) (Peng *et al.,* 2006). Sulfamethoxazole is often administrated in combination with trimethoprim, and commonly analyzed together (Gobel *et al.,* 2005). The class of tetracyclines, widely used broadspectrum antibiotics, with chlortetracycline, oxytetracycline, and tetracycline as mostly used, was detected in raw and treated sewage in many studies in the ng/L (Kim *et al.,* 2005) to mg/L concentrations (Yang *et al.,* 2003). Tetracyclines and fluoroquinolones form stable complexes with particulates and metal cations, showing the capacity to be more abundant in the sewage sludge (Alexy *et al.,* 2004; Daughton *et al.,* 1999). Some of the most prescribed antibiotics—macrolides clarithromycin, azithromycin, roxithromycin, and dehydro-erythromycin were found in various environmental matrices in a variety of concentrations from very low ng/L to few mg/L (Gobel *et al.,* 2005; Karthikeyan *et al.,* 2006).

Many active antibiotic substances were found in raw sewage matrices, including both aqueous and solid phase. The occurrence of antibiotics may promote the development of bacterial resistance, which may be stimulated by exposure to low concentrations (Jorgensen and Halling-Sorensen, 1998). (Baquero *et al.,* 2008; Kummerer, 2004) investigated that HWW is a source for undesirable constituents, such as (multi-) antibiotic-resistant bacteria.. As a consequence, occurrence of antibiotics in the aquatics environment increased our motivations to studying the antibiotic resistance phenomena.

Finally, this work is a shed of light about two principal axes: the impact of hospital wastewater on the biomass and the improvement for treating the hospital wastewater. This work is a part of many efforts affected to control and decreased the organics micropollutants in the environment.

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Chapter II

Material and Methods

1. Study area and wastewater characteristics

Activated sludge was sampled in the aeration tank of the municipal WWTP of the city (Limoges, France), (285,000 inhabitant-equivalents) which received the hospital effluents (HE), contributing to ≈2% of the total basal flow arriving in the WWTP, and the urban effluents (UE), contributing to ≈15.4% of the total basal flow arriving in the WWTP. This plant treats domestic and a very small fraction of industrial wastewater (about 10 percent) and operates advanced activated sludge treatment with an output of 47000 m^3 per day in dry weather and 81000 m^3 during rain (wastewater 47000 m^3 per day and run off 34000 m^3 per day). The sampled sludge from clarifier had an initial concentration of 3.5 to 5g.L^{-1}.

This study was realized on a 869-bed teaching hospital located on the centre of France, and which water consumption reaches 923 m^3 per day. The HE samples analyzed in this study were collected from the sewerage system which comprises only sewers from clinical activities of the hospital. The UE receives wastewater from 13 360 population equivalents which comprised mainly domestic wastewater. None HE is present in this effluent.

Average pharmaceuticals quantifications, and physic-chemicals characteristics of wastewaters and activated sludge used during the experiments are detailed in the Table 1.

Table 1: Physicochemical characteristics of the HE and UE feed wastewaters overall the study, as well as the activated sludge *inoculum* used at the beginning of the experiment for the both reactors. Standard deviation values are in brackets.

		HE	UE	AS
COD (mg.l^{-1})	Total	325.8 (117.5)	183.9 (78.3)	1120
	Soluble	188.3 (54.7)	89.5 (41.3)	120
N (mg.l^{-1})	Total	115.1 (15.0)	113.3 (11.3)	-
	Soluble	89.6 (23.1)	93.2 (11.3)	-
TSS (g.l^{-1})		0.208 (0.061)	0.143 (0.064)	3.115 (0.134)
VSS (g.l^{-1})		0.237 (0.086)	0.135 (0.067)	2.550 (0.070)

Table 2: Concentration (ng.l^{-1}) of some relevant pharmaceuticals.

Type of compound	Compound	HE	UE
Contrast Media	Iopamidol	110798 (80843)	6460 (2091)
	Ammonium diatrizoate	n/d	n/d
Antibacterials	Ciprofloxacin	455 (172)	n/d
	Clarithromycin *	n/d	n/d
	Sulphamethoxazole*	280 (70)	n/d
Antibacterials metabolite	N-acetyl sulphamethoxazole***	1051 (599)	<200ng/l
Anti-inflammatories	Naproxen**	n/d	n/d
	Diclofenac	n/d	n/d
Cytostatics	Cyclophosphamide*	290 (159)	n/d
	Ifosfamide	265 (271)	n/d
Anaesthetic	Lidocaine**	537 (560)	#DIV/0!
Anticonvulsants / tranquilisers	Carbamazepine**	114 (35)	193 (56)
Betablockers / Anti-hypertensives	Atenolol**	103 (44)	215 (49)
*LOQ 80ng/l; **LOQ 10ng/l; ***LOQ 200ng/l			

[a]The pharmaceutical compound selection has been done as the recommended priority list selected in the PILLS projec.

2. Reactors and operating conditions

2.1. Aerobic and anaerobic reactors (Batch mode)

The pilot is made of four aerobic and four anaerobic stirred reactors (Figure 1). Each reactor has a working volume of 3 litres. The reactors were filled with 2000 mL of sample sludge coming from Limoges WWTP (sludge of aerobic tank, for aerobic reactors) and 1000 ml of effluent.

The aerobic and anaerobic reactors were studied in a batch mode. Parameters that were monitored simultaneously throughout each experiment were pH, Temperature (T°C), Dissolved Oxygen (DO) and biogas volume. The pH and Temperature measurements were done using digital pH metre (Digital pH metre SUNTEX instruments, Taiwan) and dissolved oxygen were done using digital DO meter (Mettler-Toledo, SG6, Germany).

Figure 1: Photo of pilot plan with 8 aerobic and anaerobic reactors.

2.2. Conventional activated sludge system tests (CAS)

Two identical lab scales CAS (figure 2; total volume 14 L) was continuously fed with wastewaters collected each 7 day at the hospital. Wastewaters were kept in a refrigerated (4°C) and agitated tank where it was directly pumped to feed the pilots. Influent flow rate was 21.6 L.d^{-1} corresponding to a hydraulic residence time of 15.3 h^{-1} in the aeration tank. Sludge recirculation from the clarifier was maintained at 100% of feed flow rate, allowing a 60 min return activated sludge retention time in the thermal treatment reactor.

Aeration was operated by repeated aerobic/anoxic cycles (3h/3h) in order to ensure nitrification and denitrification. Air flow rate was adjusted daily in order to have a dissolved oxygen concentration between 2 and 4 mg.L^{-1} in the reactors during aerated phases (no limitation by oxygen concentration). Solids residence time (i.e. sludge age) was maintained at 15 days throughout the experiments: settled sludge was wasted every two day accordingly considering sludge losses through the effluent.
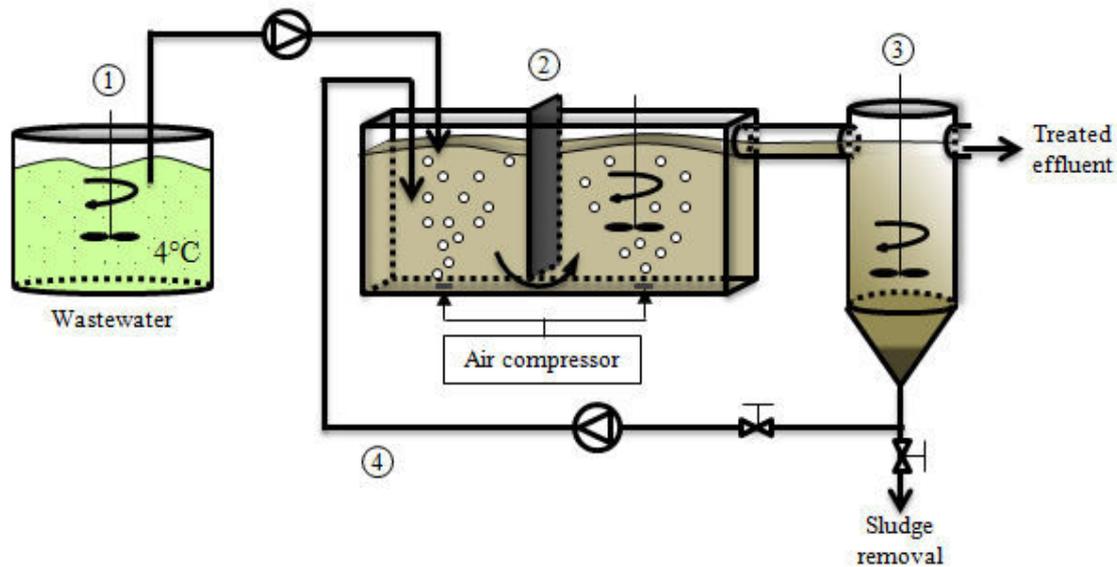
Figure 2: Description of the conventional activated sludge process used. Wastewaters were kept in a refrigerated (4°C) and agitated tank (1) where it was directly pumped to feed the pilots. Influent flow rate was 21.6 L.d^{-1}, and corresponded to a hydraulic residence time of 15.3 h^{-1} in the aeration tank (2) where aeration (2 and 4 mg.L^{-1}) was operated by repeated aerobic/anoxic cycles (2h/2h) in order to ensure nitrification and denitrification. Sludge recirculation (4) from the clarifier (3) was maintained at 100% feed flow rate. Solids residence time (*i.e.* sludge age) was maintained at 15 days throughout the experiments: settled sludge was wasted every two days accordingly considering sludge losses through the effluent.

2.3. Membrane bioreactor (MBR)

The pilot scale (Figure 3) comprised a 30 l bioreactor and U-shaped hollow fiber membrane module was immersed in the bioreactor. Hollow fibres ultra filtration used were made of polyethylene with a pore size of 0.05 µm (Mitsubishi Rayon Co., Ltd., Japan). Aeration was done through diffusers at the bottom of the reactor to provide oxygen for biomass growth as well as shear to reduce cake formation at membrane surface. Dissolved oxygen levels were maintained between 2 and 4.5mgO2/L. The membrane permeate was continuously removed by a peristaltic pump under a constant flux (1.8 L/h), constantly monitoring the trans-membrane pressure (TMP) build-up which indicates the extent of membrane fouling. The operation was stopped when the TMP reached 26 kPa because it was difficult to maintain the flux at constant level at TMP of over 26 kPa. The hydraulic retention time (HRT) ranged from 15 to 24 h. temperature was 14–20 ◦C and pH was 7–8. The sludge retention time (SRT) was around 15 days.
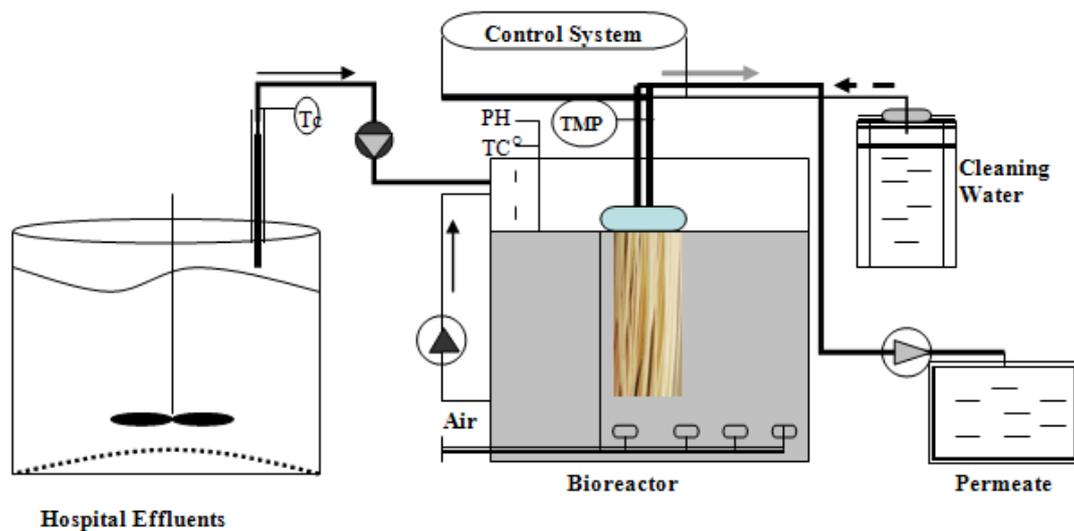
Figure 3: schematic diagram of the membrane bioreactor

Table 3: Key operational parameters of CAS and MBR systems investigated.

	HRT (h)	SRT (days)	Flow (m3/h)	Temperature (C°)	PH	Dissolved oxygen (mg/L)	Aerobic tank (L)
MBR	15-24	15-20	0,0018	16-19	7,0 - 8,0	2,0 - 4,5	30
CAS	15,3	15	0,0009	17-20	7,0-8,0	2,0-4,5	14

2.3.1. Cleaning protocol

The membrane module was systematically cleaned prior to each critical flux test. For experiments reported in situ chemical cleaning of the membranes was carried out by soaking in solution of 4g/l de Na OH + 200 ppm, contact time = 2 h with 25 C°, after that in solution of 1 % citric acid, contact time = 30 min with 25 C°, finely in solution of   HCL ph = 2, contact time = 30 min in 25 C°.

2.4. Ultrafiltration system (AS-UF)

The reactor consisted of bioreactor with a working volume of 400 L and a membrane module in an external circulation loop (figure 4). The membrane module was a Polypropylene and type of fibres cruses (UF) membrane with 1m² of surface area and pore size of 0.2µm (ALTING, MICRODYN, France). A Ruston turbine (80-120 rpm) was installed to keep the bioreactor completely mixed. An identical lab-scale cross-flow MBR was run and inoculated with activated sludge from a municipal wastewater treatment plant (dry weight, 2.5 g/L). The influent was a hospital effluent (average flux 100 L/ day). Flow of permeate was about

50 L/h. The hydraulic retention time (HRT) was 22 h, temperature was 18-20C° and pH was 6.8 to 7.9.

The sludge retention time (SRT) was around 20 days. Treatment was operated in aerobic conditions. Dissolved oxygen levels were maintained between 1 and 4.5 mg O2/ L. The aeration cycle was automatic based on tow limits. The cycle of operation was formatted automatically to 1h and 40 min (see table 2). Pressures were measured at the inlet (P1), outlet (P2), and permeate side of the membrane (P3) in order to determine the Transmembrane pressure (TMP). At constant permeate flux, TMP indicates the extent of membrane fouling and it was calculated as follows:

$$TMP = [(P1 + P2) / 2 - P3] \qquad (Eq.\ 1)$$

Fournisseur	Stohr
Activated area m²/m³	660
Diameter (mm)	12,2
Length (mm)	12
Density	0,95- 098
Materiel	PEHD
Weight (Kg/m²)	150

Figure 4: schematic of Activated sludge followed by ultrafiltration system (AS-UF).

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Table 4: Conditions of bioreactor operation

Condition of bioreactor operation	
Operating parameters	**Operating range**
Concentration of oxygen	1- 5 mg O2/L
PH	6,9 -8
T C°	16- 20
Agitation	80-120 tr/min
Volume (L)	400 L
flow of outlet (L.d-1)	1300-1700 L / j
SRT (d)	15-20 jours
HST	22 h
Aeration	Auto - 1-5 mg.O2/L
Time of presence O2 (h)	6
Flux out let (14-20°C) (L.m-2.h-1)	40-50 L/h
Mode of filtration	position horizontal- vitesse tangentielle Interne- Externe T
Tangential speed along the membrane (m / s)	0,286m/s
Type of initial treatment	Decantation
Cycle of operation	
Time of decantation	20 min
Time of transporte	20 min
Temps of filtration	20 min
Temps of alimentation	**40 min**
Volume of tank	150 L
Volume of tank the washing	150 L
Flow of pump Booster	900 L/h
flow of pomp of circulation	800-950 L/h
flow of inlet	4,25 L/ h
TMP	0,1 - 0,25 bar

3. Analytical methods

Wastewaters and sludge physic-chemical characteristic measurements were done every two day. Measurements of total and volatile suspended solids (TSS and VSS) were done according to the normalized method (AFNOR, NF T 90-105). Chemical Oxygen Demand (COD) was measured by the closed reflux colorimetric method (ISO 15705:2002), and total nitrogen (TN) was assessed using the alkaline persulfate digestion with colorimetric finish (Hach com - pany). The COD and TN were carried out on both total and soluble fraction (after samples fil- trated at 1.2µm). Ionic species in solution were determined on samples filtrated at 0.22µm using ion chromatography (DIONEX 120) according to the standard method (AFNOR, NF EN ISO 10304-1). The used detector was conducted metric, and the analytical error was ±5% (Table 5).

Table 5: Analytical methods and precision of measurement

Mesure	Method	Sample volume	Phase	Precision of measurement
COD	Method colorimetric en micro tubing (ISO 15705 :2002	2 ML	fraction totale fraction soluble	± 200 mg O2/L (± 5%) sur la fraction totale et de ± 6 mg O2/L sur la fraction soluble
N total	Micro méthode HACH	2 ml	Fraction totale fraction soluble	±5 mg
ANIONS CATIONS	Chromatographie ionique	900µl	filtration 0.22µm	-
TSS , TVS	AFNOR 1997 a Filtration 1.2µm , 105°C 12h then 550°C 2h	40 ml	Sludge total	2 %

4. Characteristics of activated sludge Flocs

4.1. Sludge characterizations

The indication of sludge (IB) or indication of Mohlman (IM) represents the volume occupied by sludge after 30 minutes of settling and returned to the concentration in SM of the test.

$$IB = [V30 /TSS]$$

With:

(IB): The indication of sludge.

V30: volume of decanter after 30 minutes of decantation.

TSS: concentration of total suspended materials.

4.2. EPS analysis

4.2.1. EPS extraction and chemical analysis

The analysis of EPS in biomass was made through a thermal extraction method. The mixed liquor of activated sludge was centrifuged at 4,000 rpm for 20 min at 4 C° in order to remove the soluble EPS from bound EPS. After collecting the soluble EPS, the remaining pellet was washed and re-suspended in saline water (0.9% NaCl solution). The extracted solution was obtained from a heat treatment at 80 C° for 1 h. The extracted solution was then separated

from the sludge solids by centrifuging under similar conditions (4,000 rpm for 20 min and T= 4C°), the supernatant obtained at this stage being referred to as bound EPS solution. The EPS is composed of many components such as protein, carbohydrate, nucleic acid, and lipids. In this study, the main component of EPS to be considered was protein and carbohydrate.

4.2.2. Analysis of total protein, humic substances and polysaccharides

Protein content, expressed in mg equivalent of bovine serum albumin per gram of VSS (mg/ L for the soluble polymer), was determined according to the method of Lowry et al. (1951) with a correction for the humic substances. Humic substances were measured with the Folin-Ciocalteau phenol reagent in the same trial as the protein by omitting the CuSO4. Results were expressed in mg equivalent of humic acid per gram of VSS (mg/ L) for the soluble polymer). Polysaccharides were determined according to the method of Dubois et al. (1956) and the results expressed in mg equivalent of glucose per gram of VSS (mg/ L) for the soluble polymer).

<u>Proteins</u>

Proteins are large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. The sequence of amino acids in a protein is defined by a gene and encoded in the genetic code.

A more sensitive method is measurement of protein with the Folin-Ciocalteau phenol (FCP) reagent (Lowry *et al.,* 1951). The first step is a burnt reaction where peptide bonds in protein react with copper in alkaline solution. The next step is a reduction of the active phosphomolybdic and phosphotungstic acids in the reagent by the copper treated protein. The colour developed is measured spectrophotometrically.

The principal disadvantage of the method of Lowry is the risk of interference from many substances. The Lowry method is slightly variable in sensitivity to various proteins but on average 27 different proteins resemble the colour developed by bovine serum albumin (Davis, 1988).

The choice of method for measuring protein in wastewater is not obvious, because none of the reviewed methods are ideal for this purpose. Considering the requirement for sensitivity and specificity for protein, the choice is between the methods of Lowry *et al.* (1951) and

Bradford, (1976). According to variability in extinction between different proteins, the Lowry method seems to be much more accurate than the Bradford method (Kamma *et al.,* 1994). Proteins were measured using the method proposed by Lowry method using BSA (Bovine Serum Albumin) as a standard protein and modified slightly by Kamma *et al.* (1994). Four reagents were prepared:

• Reagent A: 2% Na_2CO_3 in 0.1N NaOH

• Reagent B1: 0.5% $CuSO_4 \cdot 5H_2O$

• Reagent B2: 1% sodium tartrate double Na and K

• Reagent C: 48 mL Reagent A + 1mL Reagent B1 + 1mL Reagent B2

• Reagent E: 1N Folin Reagent

To start, 2.5 mL of Reagent C was added to 0.5 mL of sample, immediately mixed, and let stand for exactly 10 min at room temp. Reagent E was added (0.25 mL), the sample mixed, and incubated at room temperature for 30 min. Absorbance was measured at 650 nm, with UV – Visible spectrophotometer; model UV – 1700, Pharma-Spec, (SUIMADZU) and with bovine serum used as the standard. The measurement error is about 5 – 8% for the soluble proteins concentration and 10 to 15% for the total concentration of protein.

Carbohydrates

Carbohydrates (from hydrates of carbon) or saccharides (Greek meaning "sugar") are simple organic compounds that are aldehydes or ketones with many hydroxyl groups added, usually one on each carbon atom that is not part of the aldehyde or ketone functional group. The dominating carbohydrate sources in wastewater are starch- and dairy products, vegetables, fruit and cellulose having a monomeric composition of glucose, fructose and galactose which are all hexoses (Kamma *et al.,* 1994). Carbohydrate concentrations were determined using the phenol sulphuric method introduced by Dubois *et al.,* (1956) and modified slightly by Kamma *et al.,* (1994). Carbohydrate concentration was calculated from a calibration curve constructed using a glucose standard. Four reagents were prepared:

• Sulfuric acid, reagent grade 95.5%.

• 5% Phenol solution in water.

To start, 0.5 mL of sugar solution is pipette into a colorimetric tube, and 0.5 mL of 5% phenol is added. Then 2.5 mL of concentrated sulphuric acid is added rapidly, the stream of acid

being directed against the liquid surface rather than against the said of the test tube in order to obtain good mixing. The tubes are allowed to stand 5 minutes, and then they are shaken and placed for 5 to 10 minutes in a water bath at 25 to 30°C. Before readings are taken. The colour is stable for several hours and reading may be made later if necessary. The absorbance of the characteristic yellow-orange colour is measured at 490 nm with UV – Visible spectrophotometer; model UV – 1700, Pharma-Spec, (SHIMADZU). The amount of sugar may then be determined by reference standard curve. The measurement error is about 10 - 12% for the concentration of carbohydrates in the soluble phase and around 8 – 10% for total carbohydrates concentration.

5. Microscopic techniques

5.1. Confocal laser scanning microscopy

Bright field microscopy was carried out with an optical microscope (Olympus CX 31) equipped with a video CCD camera connected to a PC via a grabbing card. After having deposited a drop of activated sludge on a slide and spread it with the enlarged tip of a plastic pipette, the smear was let to dry before grabbing at least 100 images (magnification 100*x*) Care was taken to avoid overlapping fields. Pixel size calibration was done with a stage micrometer (160 pixels = 100 µm). The images were analyzed using the procedure described in (da Motta *et al.*, 2003) and implemented on Visilog 5 (Noésis, Saint-Aubin, France). The activated sludge structure was quantified by the ratio of the number of small fragments (fragments with an area smaller than 6.25 μm^2 which are not filaments) with respect to the total area occupied by the biomass on the image and the filaments abundance (ratio of the filament length (in pixels) to the total area occupied by the biomass on the image).

The Live/Dead®BacLightTM bacterial viability stain was used according to the manufacturer's instructions (Molecular Probes, Eugene, Oregon, USA). The kit provides a two-color fluorescence assay of bacterial viability relying on membrane integrity: viable bacteria are stained by SYTO ® 9 and fluorescent green, while damaged bacteria are stained by propidium iodide and fluorescent red. Protocol established by (Lopez et al., 2005) was performed: 1 mL of undiluted biomass suspension was mixed with 3 µL of a mixture of equal parts of SYTO® and propidium iodide. This short staining protocol allowed direct observation of the original

floc structure. No centrifugation or fixation steps were needed. Microscopic observations started 15 min after staining. Fluorescence emissions were recorded with an airy disk confocal pinhole opening and 512 x 512 images at a 0.28 μm (x,y) pixel size were obtained (pinhole 118 μm, pixel dwell time of 3.2 μs). A constant 1 μm step size in the vertical direction was used when imaging the 3D structures. Phase-contrast microscopy was also used to image the structure of the flocs. Excitation maxima for SYTO®9 and propidium iodide bound to DNA were 480 and 540 nm, respectively. SYTO®9 was excited at 488 nm and the fluorescence emission was collected in the green channel between 505 and 550 nm. The propidium iodide was excited at 543 nm and the fluorescence emission was collected in the red channel after a long pass filter at 585 nm. The EPS of the activated sludge flocs was excited at 405 nm and their auto fluorescence was collected between 505 and 550 nm in the blue channel.

PS (Polysaccharides) and PN (Proteins) staining was carried out according to the modified procedure of [14]. Bio samples were centrifuged to remove supernatant, washed twice with 1× phosphate-buffered saline (PBS) buffer (pH 7.2) and kept fully hydrated in 2 mL centrifuge tubes covered with aluminium foil. For PS staining, 100 μL of concanavalin A conjugated with tetra -methylrhodamine (Con A, 250 mg L−1, Molecular Probes, and Carlsbad, CA, USA) was first dripped onto the sample and incubated for 30 min to stain α-mannopyranosyl and α glucopyranosyl sugar residues. For PN staining, 100 μL of sodium bicarbonate buffer (0.1 M) was introduced to the sample to maintain the amine groups in non-protonated form. Subsequently, 100 μL of fluorescein isothiocyanate solution (FITC, 1 g L−1, Fluka) was supplemented and incubated for 1 h to bind to proteins. Samples were washed tow times with 1× PBS buffer after each staining stage to remove loosely bound and excess dyes. Finally, sectioned granule or biofloc samples were mounted onto microscopic glass slides for observation of the distribution of PS and PN by a confocal laser scanning microscopy equipped with an Ar–He–Ne laser unit and three barrier filters. Samples were visualized with a ×10 objective and analyzed with the start LSM image browser confocal software.

5.2. Digital image analysis

Image analysis was performed with the freely available software Image J version 1.39i including the LSM Reader plug in to open LSM5 formatted image stacks created by the microscope software. The tool Image J Analyzer 1.1, which is based on the performance of Image J and handles LSM5 formatted image stacks, was programmed for quantitative analysis. By setting a threshold, pixels with intensity below the threshold were assigned to the background. All other pixels were set to the foreground. Due to the individual image adjustment during the image stack acquisition, the threshold was chosen manually for each image stack. It has to be stressed that the pitfalls of threshold setting by the operator is well known (Staudt et al., 2004).

6. Bimolecular analyses

6.1. DNA extraction

Activated sludge was sampled weekly until the end of the experiment, for molecular biology analyses. Activated sludges (2-mL), were pelleted at 15000 g for 10 min, and total DNA was extracted using the FastDNA® spin kit for feces and the FastPrep® Instrument (MP Biomedicals), following the manufacturer's instructions. Feed wastewater samples were filtered in triplicate under vacuum through sterile 47 mm-membranes with a porosity of 0.45µm (Millipore). Total DNA was extracted from bacteria retained on the filters using the PowerWater® DNA isolation kit (MoBio Laboratories Inc.), following the manufacturer's instructions. In order to have a global view of the bacterial diversity of the feed effluents, total DNA of each sample of HE or UE were pooled to performed all bacterial diversity analyses. Before storage at -20°C, the quality of extracted DNA was verified by electrophoresis through a 0.8% (w/v) agarose gel, and their quantity was assayed in triplicate using a Nanodrop® spectrophotometer (Thermo Scientific).

6.2. PCR-DGGE experiment

The PCR-DGGE was performed as Laurent *et al.* with some modifications. Briefly, one DNA extract of each activated sludge sample was used to perform a PCR experiment using the universal bacterial primers 341F with a 40-bp GC-clamp and 518R. For the PCR, $1ng.\mu L^{-1}$ of template DNA was used in a final PCR mixture volume of 100µL. Twenty five microliters of each PCR product were loaded onto 8% (w/v) polyacrylamide gel (containing 37.5:1 of

acrylamide to bis-acrylamide) with a linear denaturant gradient ranging from 40% to 60% (of urea w/v and formamide v/v). The DGGE was performed at 60°C for 15h at 100V in 1X TAE electrophoresis buffer with D-code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). Gel images were analyzed using Quantity One Quantitation Software Version 4.6.1 (Bio-Rad Laboratories, Hercules, CA, USA).

6.3. Pyrosequencing

Pyrosequencing was done to analyse the bacterial diversity. The V3 and V4 region, of the 16S rRNA encoding gene were chosen to analyse the bacterial diversity using the bacterial universal primers 339F (CTCCTACGGGAGGCAGCAG) and 339R (TTGTGCGGGCCCCCGTCAATT) targeting the V3 and the V4 variable regions of the 16S rRNA encoding gene. The pyrosequencing as well as the PCR were conduct by the Molecular research LB lab (http://www.mrdnalab.com/) according to standard laboratory procedures using a 454 FLX Sequencer (454 Life Sciences, Branford, CT, USA). The detail methods and the analyse pipeline, as well as results are presented in the SI.

6.4. Diversity analyses

For each samples rarefaction analyses were performed using past (PAleontological Statistics v1.60) software from http://folk.uio.no/ohammer/past/. The Primer6 (Plymouth Routines In Multivariate Ecological Research, version 6.1.6) software was used to performed diversity indices calculation (Bray-Curtis, Chao1 richness and Shannon diversity indexes), and clustering analysis coupled to two-dimensional non-metric multidimensional scaling ordination (2D-nMDS) based on Bray Curtis similarity taking into account for the DGGE pattern, the presence or absence of band and their relative intensity, and for the pyrosequencing the relative abundance of OTU with a cutoff at 3% of sequence identity was considered.

6.5. Quantitative PCR protocol

Quantitative PCR (qPCR) assays were targeted at *intI1* gene of the class 1 RI from total extracted DNA. The quantification of the rRNA 16S encoding gene was performed in a SYBR green assay using the universal primer 338F and 518R targeting the 16S rRNA gene of Eubacteria as described previously. Assays were performed in triplicate with a MX3005P real-time detection system (Stratagene®). In order to avoid qPCR inhibitor effects total DNA

samples were diluted to the convenient dilution for which quantifications were not affected. The statistical hypothesis test Mann-Whitney was performed using the StatView$^©$ 5.0 software (SAS institute Inc.), a p-value <0.05 was considered to be significant. For an accurate quantification, the *intI*1 gene and the 16S rRNA encoding genes were embedded in a single standard plasmid. A full standard curve, made up between 10^3 and 10^8 copy number for standards, was included in duplicate in each qPCR run. The total estimated bacterial cell number was recovered with 16S rRNA encoding genes quantification. Based on the Ribosomal RNA Database, the number of the 16S rRNA encoding genes per bacteria is currently estimated to 4.1. Therefore all 16S rRNA quantifications have been divided by 4.1 to estimate the total bacterial load. The relative abundance of class 1 RI was calculated by dividing their concentration by the estimated bacterial concentration.

7. Dosage the pharmaceuticals compounds in wastewaters

7.1. Sample preparation

Waters were centrifuged at 10000g for 10min and the supernatant was acidified to pH=3 using formic acid (Sigma). This acidified solution was passed through SPE column *Oasis HLB 3 cc Cartridge* (Waters), under vacuum. The SPE column was air dried and stored at -20C. HLB cartridges were first washed with 6ml water, then eluted with 8ml CH_3CN (Fisher)/MeOH (Fisher), containing 0.1% formic acid. They were then dried down ready for analysis. Dried down samples were re-constituted in CH_3CN/H_2O (distilled) 30/70. Internal standards were added to afford a concentration of 5ug/l. Calibration standards were prepared and run at the following concentrations ranges: Atenolol, Lidocaine, Ciprofloxacin, Cyclophosphamide, Sulphamethoxazole, Ifosfamide, N-acetyl sulphamethoxazole 10 – 5000 ug/ml. Iopamidol, Ioprimide and Diatrizoate 40 – 20000 ug/ml. Criteria was minimum 4 points on the line +/- 20%.

7.2. Detection

The LC method was a gradient from 1% orgainic to 99% organic over 54 mins. Flow rate was 200ul/min and injection volume was 10ul. The column oven was set to 30°C. Column was an *Atlantis dC18 3um 2.1 x 150mm* (Waters). A binary pump *Agilent 1100 series LC system* with electrospray ionisation and a detector *Esquire 3000 plus Ion Trap mass spectrometer* from *Bruker Daltonics* was used for the detection.

7.3. Dosage the PPCP in the wastewater

Tow different analytical methods were applied to determine the concentration levels of the PPCPs in the wastewaters samples. Analyses were performed by the IANESCO. Water samples were enriched by liquid-solid phase (SPE) by using Osis HLB cartridges (6ml, 200mg) from waters. The SPE extracts were injected in liquid chromatography- mass spectrometry (LC-MS/MS). Acquisition was performed in selected reaction monitoring (SRM) mode and tow transitions (quantification, confirmation) were obtained for each compound.

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Chapter III

Results and Discussion

Evaluation des substances polymériques extracellulaires par microscopie confocale dans un réacteur à boues activées et les un bioréacteur membranaire lors du traitement d'un effluent hospitalier

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La mise en présence d'un effluent contenant des molécules toxiques, comme les composés pharmaceutiques ou les détergents, avec une biomasse bactérienne peut engendrer différentes modifications structurelles et biochimiques de cette dernière. Ainsi certaines molécules peuvent être létales pour des populations bactériennes, d'autres déclencher la défloculation des flocs bactérien ou, au contraire, promouvoir la mise en place de protections comme la génération de polymère structurant ces mêmes flocs bactériens.

Afin de suivre ces impacts potentiels, des techniques d'évaluations des substances polymériques extracellulaires (EPS)et de viabilité cellulaire ont été testées. La microscopie confocale (CLSM : confocal laser scanning microscopy) combinée avec des indicateurs fluorescents de viabilité, a été utilisée parallèlement aux méthodes d'analyses biochimiques lors du traitement biologique d'effluents hospitaliers. Trois pilotes, une boue activée classique, un bioréacteur à membranes immergées (MBR) et un bioréacteur à membranes externe, ont été installés et exploités pendant 65 jours. Les concentrations de micropolluants pharmaceutiques mesurées allaient de quelques ng/L au mg/L.

Des échantillons de flocs prélevés dans chacun des systèmes ont été marqués par une solution de concanavaline A et ethylrhodamine, d'une part et d'isothiocyanate de fluorescéine d'autre part, pour caractériser, au cours du temps, les évolutions des substances polymériques extracellulaires. Un indicateur fluorescent de la viabilité cellulaire (Kit Baclight® viabilité bactérienne, Moléculaire Probes) a également été utilisé afin de suivre la viabilité cellulaire durant le temps de l'expérimentation. Les échantillons ainsi marqués ont été observés par microscopie confocale à balayage laser.

Les analyses biochimiques classiques d'EPS ont été effectuées au moyen d'un procédé d'extraction thermique et les résultats comparés à ceux obtenus par observation microscopique.

L'analyse des résultats obtenus aboutit aux conclusions suivantes:

- les efficacités de traitement de l'effluent hospitalier, en terme de paramètre classique de pollution, sont bons dans les 3 cas ; il n'y a pas d'effet notable d'inhibition de l'activité bactérienne. Les a membrane permettent cependant les meilleurs taux d'élimination.
- On note une évolution des concentrations en EPS différentes aux cours du temps pour les trois procédés, pourtant alimentés par le même effluent. Les concentrations les plus importantes sont obtenues dans le MBR. Différentes hypothèses sont avancés pour expliquer ce phénomène, notamment l'importance de l'âge des boues.
- l'évolution de la mortalité cellulaire ne montre pas une surmortalité significative mais des distributions dans les flocs variables.
- Les images obtenus par CLSM traités par analyse statistique, ont montré une bonne relation entre les deux modes d'analyses validant ainsi le suivi des évolutions des concentrations au cours du temps des EPS et de la viabilité cellulaire, notamment en réacteur membranaire immergé. Cette technique sera utilisée pour le suivi des EPS dans les différents procédés utilisés lors de nos travaux.

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Evaluation of the extracellular polymeric substances by confocal laser scanning microscopy in conventional activated sludge and advanced membrane bioreactors treating hospital wastewater

Mousaab Alrhmoun, Claire Carrion, Magali Casellas and Christophe Dagot

ABSTRACT

Confocal laser scanning microscopy (CLSM) combined with fluorescent viability indicators, was used in this study to investigate the impact of hospital wastewaters on floc structure and composition. In this work, three pilot-scale projects, two membrane bioreactors (MBRs) with a submerged or external membrane bioreactor and a conventional activated sludge, were installed and operated for 65 days. They were fed with an influent sampled directly from the hospital drainage system, which contained micropollutant concentrations ranging from ng/L to mg/L. Samples of flocs were observed using CLSM to characterize the extracellular polymeric substances (EPS) stained with concanavalin A–tetra methylrhodamine and fluorescein isothiocyanate solution and combined with a fluorescent viability indicator (Baclight® Bacterial Viability Kit, Molecular Probes), allowing visualization of isolated stained cells in the three-dimensional structure of flocs (damaged or not). The results of CLSM of the sludge composition were compared with classical biochemical analysis of EPS made through a thermal extraction method. The results showed a good relation between these analyses and the statistical treatment of microscopic pictures.

Key words | confocal laser scanning microscopy, EPS, extracellular polymeric substances, MBR, membrane bioreactors

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INTRODUCTION

In biological wastewater treatment systems, occurrence of extracellular polymeric substances (EPS), a complex high molecular weight mixture of polymers bound to bacterial flocs, has been observed using various electron microscopy techniques (Sheng et al. 2010). This often leads to decreased performance of the wastewater treatment plant, to reduce settling properties of activated sludge flocs in the secondary clarifier and, in the worst case, to impact on the water quality (Schmid et al. 2003). EPS are composed of a wide variety of organic materials, including polysaccharides, proteins, nucleic acids, lipids and humic substances. In an activated sludge system, EPS mainly contain two forms, soluble EPS (SEPS) and bound EPS (BEPS) according to their location around the cell (Wingender et al. 1999; Rosenberger et al. 2002).

In recent years, membrane bioreactors (MBRs) have given rise to increased attention in the field of wastewater

technologies (Zator et al. 2007). An important problem in MBR treatment is the fouling of the membrane and that leads to a decrease in performance of the MBRs. The fouling properties of the membrane can be affected by a number of factors, such as the amount and the composition of EPS, the distribution in the sludge and the structure of flocs. The relative importance of these factors is, however, not well understood (Ramesh et al. 2006).

Recently, among the various techniques that exist for the visualization and distribution of EPS in activated sludge or sorbed on the MBR, confocal laser scanning microscopy (CLSM) was found to be a powerful tool used on the micrometre scale (Le-Clech et al. 2007; Wang & Wu 2009). CLSM could be used to observe fully hydrated samples to obtain the original shapes and structures of EPS (Zhang & Bishop 2001). After staining by various fluorescence probes, the

spatial distributions of carbohydrates, proteins and nucleic acids in EPS can also be obtained by CLSM, in addition to conventional chemical colorimetric analyses which can be used to quantify their contents in EPS (Raunkjær *et al.* 1994).

A comparison between the two methods for carbohydrates, protein and humic-like substances content determination in EPS showed that the two methods yielded similar results, but that the coefficient of time and the analyses cost for the CLSM method was lower than that for the conventional chemical colorimetric method. The purpose of this investigation was to evaluate the suitability of images from the microscopic technique (CLSM) as a basis for quantitative image analysis (Sheng *et al.* 2010).

MATERIALS AND METHODS

Reactor configuration and operating conditions

Three laboratory-scale systems, submerged (SMBR) or external membrane bioreactors (EMBR) equipped with a polypropylene membrane module and a type of hollow fibre membrane, and a conventional activated sludge system (CAS), were used to evaluate the role of the process on the formation of EPS compounds and on the performances of the three configurations in treating hospital wastewater.

Analytical methods

Physico-chemical characteristic measurements on the wastewater and the sludge were carried out every 2 days. Measurements of total and volatile suspended solids (TSS and VSS) were performed according to the normalized method (NF T 90-105; AFNOR 1997). Chemical oxygen demand (COD) was measured by the closed reflux colorimetric method (ISO 15705:2002; ISO 2002), and total nitrogen (TN) was assessed using alkaline persulfate digestion with colorimetric finish (Hach company). The COD and TN measurements were carried out on both total and soluble fractions (after samples had been filtered at 1.2 μm). Ionic species in solution were determined on samples filtered at 0.22 μm using ion chromatography (DIONEX 120) according to the standard method (AFNOR, NF EN ISO 10304-1). The analytical error was ±5%.

EPS extraction and chemical analysis

The analysis of EPS in biomass was made through a thermal extraction method. Protein content was determined according to the method of Lowry *et al.* (1951) and the polysaccharides were determined according to the method of Dubois *et al.* (1956).

Confocal laser scanning microscopy – EPS staining and visualization

SYTO® 9 BacLight™ bacterial stains was used according to the manufacturer's instructions (Molecular Probes, Eugene, Oregon, USA). The kit provides a three-colour fluorescence assay of bacteria relying on membrane integrity: viable bacteria are stained by SYTO® 9 and fluoresce green, while damaged bacteria are stained by propidium iodide and fluoresce red. The protocol established by Lopez *et al.* (2005) and Baker & Inverarity (2004) was performed: 1 mL of undiluted biomass suspension was mixed with 3 μL of a mixture of equal parts of SYTO® 9 and propidium iodide. This short staining protocol allowed direct observation of the original floc structure and the time-lapse microscopy. Microscopic observations started 15 min after staining. Excitation maxima for SYTO® 9 and propidium iodide bound to DNA are 480 and 540 nm, respectively (Reynolds 2002). A Zeiss LCM 710 NLO confocal microscope was used for the image series. The band width of the detected fluorescence wavelengths has been optimized to uniquely channel the maximum emission in sequential mode to avoid potential interference (502–530 nm) for SYTO® 9 and (600–630 nm) for propidium iodide.

Step size was determined by choosing start and end points in the *z*-direction of the flocs, and by then selecting a number of optical sections.

Polysaccharides (PS) and proteins (PN) staining was carried out according to the modified procedure of Chen *et al.* (2007). Bio-samples were centrifuged to remove supernatant, washed twice with phosphate-buffered saline (PBS) buffer (pH 7.2) and kept fully hydrated in 2 mL centrifuge tubes covered with aluminium foil.

For PS staining, 100 μL of concanavalin A conjugated with tetramethylrhodamine (Con A, 250 mg/L; Molecular Probes, Carlsbad, CA, USA) was first dripped onto the sample and incubated for 30 min to stain α-mannopyranosyl and α-glucopyranosyl sugar residues. For PN staining, 100 μL of sodium bicarbonate buffer (0.1 M) was introduced to the sample to maintain the amine groups in non-protonated form. Subsequently, 100 μL of fluorescein isothiocyanate solution (FITC, 1 g/L, Fluka) was supplemented and incubated for 1 h to bind to proteins. Samples were washed twice with 1× PBS buffer after each staining stage to remove loosely bound and excess dyes.

Finally, sectioned granule or biofloc samples were mounted onto microscope glass slides for observation of the distribution of PS and PN by CLSM. The image acquisition settings, such as laser intensity, numerical aperture, gain and offset settings were adjusted according to Toh *et al.* (2003). Samples were visualized with 10× and 100× objectives and analyzed with the start LSM image browser confocal and Image J software.

Digital image analysis

Image analysis was performed with the freely available software Image J version 1.39i including the LSM-Reader plug. The tool Image J Analyzer 1.1, which is based on the performance of Image J and handles LSM5 formatted image stacks, was programmed for quantitative analysis. By setting a threshold, pixels with intensity below the threshold were assigned to the background. All other pixels were set to the foreground. Due to the individual image adjustment during the image stack acquisition, the threshold was chosen manually for each image stack. It has to be stressed

that the pitfalls of threshold setting by the operator are well known (Yang *et al.* 2001; Staudt *et al.* 2004).

RESULTS AND DISCUSSION

Performance of MBR systems and CAS in treating the organic pollutants

A comparison between the three reactors was made for TSS and VSS, COD and nitrogen removal rates. The results in Table 1 showed that the removal efficiencies of COD, TN, and TSS, in EMBR were 87.9, 91.1 and 99.6%, respectively, compared with 80.5, 79 and 93.6%, respectively, in SMBR and 78, 85.4 and 93.4% in CAS (Table 1). The MBR system is able to achieve COD removal by both physical and biological mechanisms.

Analysis of EPS

It has been generally believed that EPS can mediate both bacterial cohesion and adhesion. Hence, EPS, especially PS/PN, have a decisive role in building and keeping the structural integrity of a microbial community (Liu *et al.* 2004). At low solid residence time (SRT) (15 days), the concentrations of all the compounds measured in MBR sludge supernatant are higher than those with CAS until 45 days of exposure (Figure 1). These results could be explained by different assumptions: (1) first it could be assumed that a 'low' SRT (lower than 30 days) is not sufficient to degrade

Table 1 | Stabilized COD, N and SS removal efficiencies for AG-MBR and SG-MBR

Efficiency of removal (%)	TSS	VSS	Total COD	N	Soluble COD	N
EMBR	99.6	97.5	87.9	91.1	86.9	90.5
SMBR	93.6	87.8	80.5	79.0	71.6	85.4
CAS	93.4	87.2	78.0	85.4	72.8	85.5
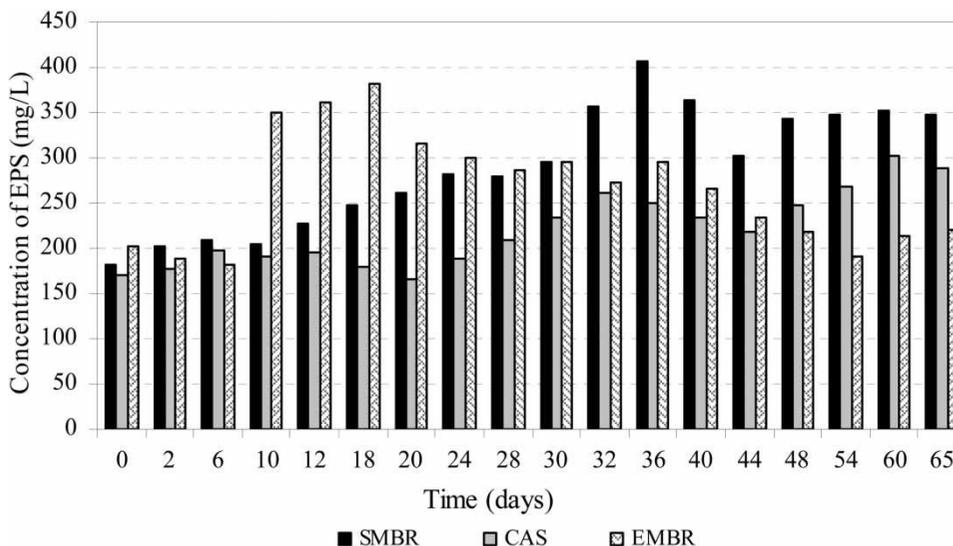
Figure 1 | EPS concentration in the mixed liquor for SMBR, EMBR and CAS systems.

all the organic compounds, which are accumulated in the system because of external membrane retention; (2) the presence of more dispersed organisms in MBR probably also aid the degradation of molecules in the supernatant due to reduction of mass transfer limitation; (3) increase of SRT could also enhance the development of slowly growing populations, which are able to use some macro-molecules (polysaccharides and proteins) as substrate; (4) finally, if it is assumed that the quantified organics (PN and PS) are principally composed of microbial products, it can be supposed that non-flocculating bacteria produced less biopolymer, which is a known flocculating agent.

EPS analysis with confocal laser scanning microscopy

Live/dead assessment within mixed microbial flocs

Propidium iodide (PPI) was used to stain dead cells and extracellular SYTO® 9 was used to stain live cells in the sample. In general, for all floc samples, the SYTO® 9 and PPI signals were distributed throughout the flocs sections. However, the intensity of the two signals varied from one region of a floc to another, probably due to the differential number and localization of live versus dead cells within the flocs.

CLSM images (Figure 2) reveal that there is an increase in blue signal intensity during the time of observation and it

is much more significant in the SMBR compared with the CAS. These modifications were attributed to a protection mechanism of the bacteria against toxic effluent (Avella *et al.* 2010). EPS might act as protective barriers against toxic substances, e.g. heavy metals or certain biocides (disinfectants and antibiotics).

Stacks of images were imported into the image software to mathematically compute relative intensities of the SYTO® 9 and PPI signals, and to calculate the relative percentage of live cells per floc.

Evolution of EPS compositions within a mixed microbial community

Figures 3 and 4 show that the visualizations of flocs collected in the EMBR reactor after 2, 20, 45, and 65 days of exposure time to the hospital effluent.

In general, through visual inspection, an increase in SEPS and BEPS was observed, especially in the first days between 2 and 20 days of exposure. This finding is in accordance with our chemical analysis which shows increasing concentration of SEPS and BEPS (PS, PN and humic-like substances) with time up to 20 days (see Figures 1, 3 and 4). Through the fluorescence indications at the start of the study (from 2 to 20 days of exposure to hospital effluents) it were observed that the polysaccharides and the humic-like substance compounds
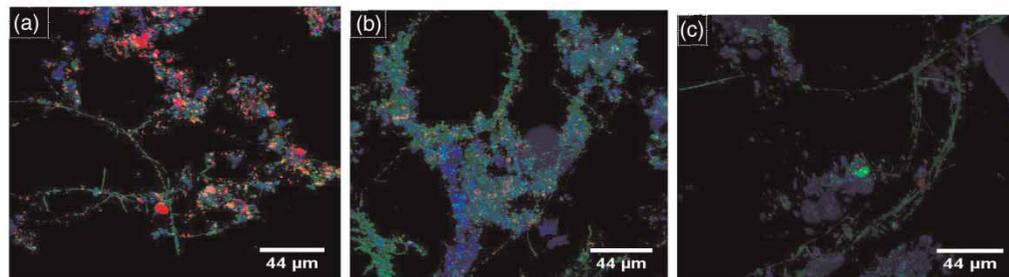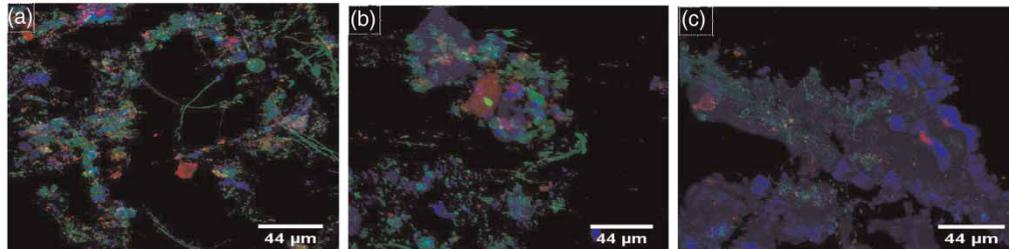
Figure 2 | CLSM image of live cell distribution within CAS and SMBR flocs. Flocs were stained with SYTO® 9 for total available DNA (viable bacteria; green) and stained with PPI for DNA of dead cells and EPS DNA (dying bacteria; red). Images obtained at x100 magnification. These representative images are based upon the examination of 5–10 flocs per sample. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.

Label	Area	Mean
Red	50625.003	19.702
Green	50625.003	1.247
Blue	50625.003	27.053
(R+G+B)/3	50625.003	16.000
0.299R+ 0.587G+0.114B	50625.003	9.705

Label	Area	Mean
Red	50625.003	23.213
Green	50625.003	31.392
Blue	50625.003	49.012
(R+G+B)/3	50625.003	34.535
0.299R+ 0.587G+0.114B	50625.003	30.964

Label	Area	Mean
Red	50625.003	46.362
Green	50625.003	0.408
Blue	50625.003	35.379
(R+G+B)/3	50625.003	27.382
0.299R+ 0.587G+0.114B	50625.003	18.133

Label	Area	Mean
Red	50625.003	18.115
Green	50625.003	2.982
Blue	50625.003	13.466
(R+G+B)/3	50625.003	11.516
0.299R+ 0.587G+0.114B	50625.003	8.710

Figure 3 | CLSM images of the BEPS distribution within EMBR flocs. Images were obtained at x10 magnification. FITC staining universal protein is in green and ConA staining α-mannopyranosyl and α glucopyranosyl is in red. Images are representative of 5–10 flocs examined. Images (a, b, c), (d, e, f), (g, h, i), (j, k, l) are for 2, 20, 45 and 65 days, respectively. (1), (2), (3) represent the distribution of the EPS constituent versus the time in the sludge from the experimental tests. In right-hand boxes, 'Red' denotes polysaccharides, 'Green' denotes proteins, and 'Blue' denotes humic-like substances. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.

increased in the bound phase (Figure 3). Then, a stabilization in polysaccharides concentration and a decrease in concentration of proteins were observed in both phases (bound and soluble) of sludge samples after more than 40 days.

The chemical analysis of the soluble and supernatant EPS matrix in the EMBR shows a slight trend towards a small increase in carbohydrate concentrations and a decrease in protein concentrations after the first 20 days of

Label	Area	Mean
Red	50625.003	17.822
Green	50625.003	17.301
Blue	50625.003	9.458
(R+G+B)/3	50625.003	14.856
0.299R+ 0.587G+0.114B	50625.003	16.583

Label	Area	Mean
Red	50625.003	12.017
Green	50625.003	0.621
Blue	50625.003	16.426
(R+G+B)/3	50625.003	9.686
0.299R+ 0.587G+0.114B	50625.003	5.829

Label	Area	Mean
Red	50625.003	8.130
Green	50625.003	0.542
Blue	50625.003	27.557
(R+G+B)/3	50625.003	12.075
0.299R+ 0.587G+0.114B	50625.003	5.886

Label	Area	Mean
Red	50625.003	43.447
Green	50625.003	76.256
Blue	50625.003	30.918
(R+G+B)/3	50625.003	50.208
0.299R+¨ 0.587G+0.114B	50625.003	61.277

Figure 4 │ CLSM images of the SEPS distribution within EMBR flocs. Images were obtained at x10 magnification. FITC staining universal protein is in green and ConA staining α-mannopyranosyl and α glucopyranosyl is in red. Images are representative of 5–10 flocs examined. Images (a, b, c), (d, e, f), (g, h, i), (j, k, l) are for 2, 20, 45 and 65 days, respectively. In right-hand boxes, 'Red' denotes polysaccharides, 'Green' denotes proteins, and 'Blue' denotes humic-like substances. The full colour version of this figure in available online at http://www.iwaponline.com/wst/toc.htm.
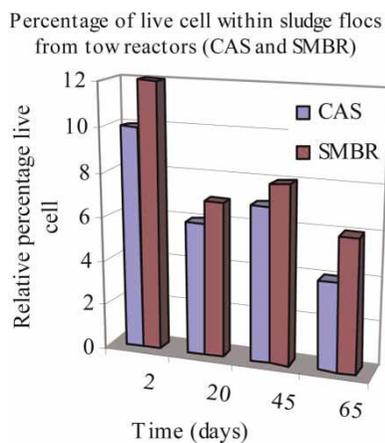
Figure 5 │ Relative number of live cells in two reactors (CAS and SMBR). The percentage was determined by measuring fluorescent intensities of SYTO® 9, which labels all DNA in a sample, and PPI, which labels DNA from cells with compromized membranes and extracellular DNA. The calculation is based on measurements of 5–10 flocs from each sampling site.

the study. The lower signal intensity at the exterior of the flocs (soluble) compared to the interior of flocs (bound) can be related to the loss of packing of EPS.

A dynamic high and low signal observed on the two sides of the central peak was possibly due to the porosity and filamentous bacteria on the exterior of the flocs. Lower intensities in the FITC could be due to fewer binding sites caused by lower concentration of these EPS constituents.

Statistical analyses

Stacks of images were imported into the J image software to mathematically compute relative intensities of the SYTO® 9 and PPI signals, and to calculate the relative percentage of live cells per floc. The relative distribution of dead/live

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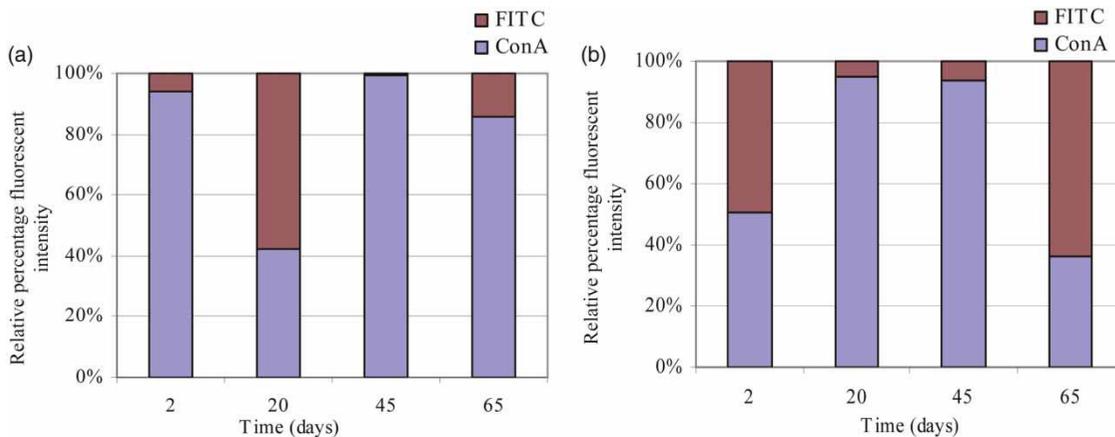
Figure 6 | Percentage of protein and carbohydrate intensity versus the time within EMBR flocs for the bound phase (a) and soluble phase (b) ($n = 5$).

cells is shown in Figure 2 and relative percentages can be observed in Figure 5 for two sites.

Five or more flocs from each sampling site were examined. We can observe the SMBR samples had the most intact cells, followed by CAS. This may indicate the overall health and well-being of cells within flocs of different origin and give an indicator on the relation between concentration of EPS and the live cells of bacteria.

The settleability and physicochemical parameters are attributed to the PN/PS ratio. The PN/PS ratios were analyzed via the bulk extraction method for the sludge samples in both phases (bound and soluble) in EMBR and was found to be from 0.3 to 1.3 in both phases.

For the sludge samples analyzed through microscopic analysis, however, the calculated PN/PS ratios were between 0.05 and 1.3 at the bound phase.

The bulk analysis revealed that the bulk EPS constituents of all sampling sites were not significantly different according to PN/PS ratios examined through the microscopic method. The relation between the intensity of the PN (FITC) and the PS (Con A) with the relative percentage fluorescent intensity versus time is reported in Figure 6.

CONCLUSIONS

It can be concluded that CLSM, in combination with image analysis, is a powerful method for direct determination of the EPS distribution, heterogeneity factors and the structure of activated sludge flocs. We have also found that there is a good correlation between the chemical analyses of EPS and the statistical treatment of microscopic pictures.

In addition, significant and specific changes of the EPS compounds, and in the microbial population structure of original flocs, could be observed in the CAS and MBR systems treating hospital effluent.

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Dynamique de la morphologie de flocs bactériens, de la diversité bactérienne, et du contenu en intégrons au cours du traitement d'effluents hospitaliers par boues activées.

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Comme dans l'étude précédente, l'objectif de cette partie du travail était de continuer à expérimenter la faisabilité de techniques d'évaluation de la modification des écosystèmes épuratoires soumis à des effluents hospitaliers. Dans ce cas, nous nous sommes intéressés, en collaboration avec d'autres équipes de l'université, à la composante microbiologique du floc bactérien dans un procédé à boue activé traitant soit un effluent urbain, collecté sur un réseau non impacté par un centre de soins, soit un effluent hospitalier (CHRU Limoges).

Les résultats du fonctionnement de deux pilotes identiques, alimentés avec l'un et l'autre des effluents ont été comparés. En terme de performance épuratoire, peu de différence a été notée, excepté pour le paramètre fraction organique des boues et DCO soluble pour l'effluent hospitalier, révélant la possibilité d'une production plus importante d'EPS. Le suivi par analyse d'images a mis en relief une forme de défloculation avec une quantité de filament plus importante lors du traitement de l'effluent hospitalier. Ces résultats ont été confirmés par microscopie confocale à balayage avec marquage des EPS et de la viabilité cellulaire. L'intérêt du suivi de l'évolution dynamique des flocs par CLSM est également confirmé.

L'impact des effluents hospitaliers sur les populations bactériennes a été abordé dans ce travail selon 2 axes : d'une part la recherche des intégrons de résistance et d'autre part par une analyse métagénomique des communautés bactériennes présentes.

Les intégrons ont été suivis car constituent un système génétique de capture et d'expression de gènes en réponse à un stress. Ils sont

notamment impliqués dans la dissémination de la résistance aux antibiotiques. Il est montré ici que l'alimentation de nos pilotes de traitement en effluent hospitalier provoque une augmentation de l'abondance relative en intégrons, comparativement au pilote alimenté par des eaux urbaines classiques.

L'analyse métagénomique comparative des communautés bactériennes a montré que le traitement de l'effluent hospitalier a entraîné l'introduction de Pseudomonas spp. dans la communauté bactérienne.

L'analyse croisée des résultats aboutit à la conclusion que les effluents hospitaliers impactent les populations bactériennes classiques des bassins de boues activée en induisant

- sur le plan physique, une érosion des flocs,
- sur le plan biochimique, une augmentation de la production d'EPS
- sur le plan biologique, une modification de la population avec l'introduction de Pseudomonas spp., connu pour ces potentialités à produire des EPS
- sur le plan du risque, une modification des potentialités, liées également à Pseudomonas spp., à échanger des déterminants génétiques (RIS) impliqués dans l'acquisition de la résistance aux antibiotiques.

Dynamic Assessment of the Floc Morphology, Bacterial Diversity, and Integron Content of an Activated Sludge Reactor Processing Hospital Effluent

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Ⓢ *Supporting Information*

ABSTRACT: The treatment of hospital effluents (HE) is a major concern, as they are suspected of disseminating drugs and antibiotic resistance determinants in the environment. In order to assess HE influence on wastewater treatment plant biomass, lab-scale conventional activated sludge systems (CAS) were continuously fed with real HE or urban effluent as a control. To gain insights into the main hurdles linked to HE treatment, we conducted a multiparameter study using classical physicochemical characterization, phase contrast and confocal laser scaning microscopy, and molecular biology (i.e., pyrosequencing) tools. HE caused erosion of floc structure and the production of extracellular polymeric substances attributed to the development of floc-forming bacteria. Adaptation of the sludge bacterial community to the HE characteristics, thus maintaining the purification performance of the biomass, was observed. Finally, the comparative metagenomic analysis of the CAS showed that HE treatment resulted in an increase of class 1 resistance integrons (RIs) and the introduction of *Pseudomonas* spp. into the bacterial community. HE treatment did not reduce the CAS process performance; nevertheless it increases the risk of dissemination into the environment of bacterial species and genetic determinants (RIs) involved in antibiotic resistance acquisition.

Macroscopic | Microscopic | Molecular

Does hospital effluent treatment affect activated sludge biomass and antibiotic resistance?
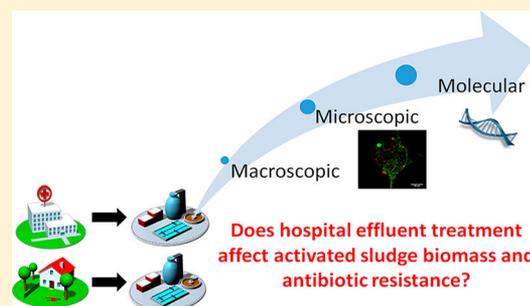
INTRODUCTION

During the past decade, several studies and international research programs (POSEIDON, PILLS, KNAPPE) have worked on the contribution of medical care activities to environmental pollution. These anthropogenic activities produce effluents that contain pharmaceuticals and antibiotic resistant determinants (ARD), which are designated as emerging contaminants by some of the studies. Several ecotoxicological surveys[1−4] showed that pharmaceuticals have adverse effects on the receiving environment, while ARDs are linked to the emergence of multidrug resistant bacteria.[5]

Hospital effluents (HE) are characterized by a large diversity of hazardous chemicals such as antibiotics, anesthetics, cytotoxic agents, disinfectants, heavy metals, and iodized X-ray contrast media.[6,7] Concentrations of pharmaceuticals in HE are most often in the range of $\mu g \cdot L^{-1}$, and a study comparing analytical measurements of 73 pharmaceuticals compounds in a

HE and an urban effluent (UE) wastewater treatment plant (WWTP) found that pharmaceutical concentrations in influents were 7-fold higher on average in the HE than in the UE. Pharmaceuticals compounds of the highest concentrations in HE included the following: analgesics/anti-inflammatories, lipid regulators, and antibiotics.[8] The influence of antibiotics on bacterial communities, in terms of impact on the microbial ecosystem equilibrium, or on the ARD content, raises specific sanitary issues, even if environmental concentrations of antibiotics are often too low to exert a direct selective pressure on bacterial communities.[9] However, *in vitro* studies have reported that subinhibitory concentrations of antibiotics could

impact the behavior of bacterial communities. They could influence mutational, recombinational, and horizontal gene transfer rates in bacteria[10] and could increase the emergence of antibiotic resistant bacteria.[11,12] Furthermore HE exhibit high concentrations of (*i*) opportunistic pathogens, such as *Pseudomonas aeruginosa*, (*ii*) antibiotic resistant bacteria of clinical interest, such as multidrug resistant *E. coli* or vancomycin and ciprofloxacin resistant *Enterococcus* spp., and (*iii*) antibiotic-resistance genes.[13−20] The complex relationship between antibiotic-resistance profiles of bacteria from HE and their downstream environments is mainly the result of horizontal transfers of antibiotic resistance genes contained by bacterial genetic elements, i.e. plasmids, transposons, resistant integrons.[21] These genetics elements often harbor more than one resistance gene, conferring multidrug resistance. Resistant integrons (RIs) are genetic elements able to acquire and exchange genes embedded within cassettes.[22] Five classes of RIs have been described, and class 1 is the most described and the most frequently found. Class 1 RIs are widespread in the environment and are mainly associated, in a medical context, with clinical antibiotic resistance in gram-negative bacteria.[23,24] In HE, it was found that more than 50% of antibiotic-resistant *Enterobacteriaceae* contained class 1 RIs.[25] They have been used for the evaluation of the prevalence of multidrug resistance in sludge from WWTP.[9,26]

Most HEs are discharged into municipal sewers connected to urban WWTPs without specific treatment aimed at bacterial- or micropollutants; the resultant impacts on the associated biological systems and, more generally, on WWTP performance raise questions about the management of these effluents.[27]

Currently, the most common WWTP types involve biological processes, which are based on the ability of endogenous microorganisms to biologically oxidize organic pollutants. In these conventional activated sludge (CAS) processes, the bacterial population is organized in macroscopic structures (floc), which are made up of macromolecules denoted extracellular polymeric substances (EPS).[28] This floc organization enables gravity sedimentation, which is a principal monitoring parameter in WWTP management. To date, the influence of HE on the biological biomass involved in the wastewater purification process has been poorly investigated in terms of the floc structure, the bacterial metabolic efficiency, or the microbial ecosystem equilibrium. Only some authors have reported the negative impact of elevated concentrations of individual antibiotics or synthetic mixtures of pharmaceuticals on the structure, activity,[29,30] and bacterial community within the WWTP biomass.[31]

In this study, we examined the treatment of a HE with a CAS system, focusing on overall performance, biomass structure, bacterial diversity, and antibiotic resistance determinants. The same treatment applied to an UE was used as a control. The originality and importance of this study lie in its multiscale approach, giving an overview of the impacts of a real HE on several major aspects of the CAS process and ARD dissemination.

■ MATERIALS AND METHODS

Conventional Activated Sludge Reactors: Tests and Analytical Methods. Two identical lab-scale CAS reactors comprising an activated sludge reactor and a settling tank (total volume 14 L) were continuously fed with HE or UE until steady-state was reached. Total suspended solids (TSS), volatile suspended solids (VSS), total/soluble chemical oxygen demand (COD), total/soluble total nitrogen (TN), and the sludge volume index (SVI) were continuously monitored on alternate days. The reactor settings and parameters and a complete description of the collection sites and effluents characteristics (including selected pharmaceutical compounds) are provided in the Supporting Information (SI).

Microscopy. Bright field microscopy was conducted using an Olympus CX 31 equipped with a video CCD camera connected to a PC via a grabbing card. After depositing a drop of sludge on a slide and spreading it with the enlarged tip of a plastic pipet, the smear was left to dry before grabbing at least 100 images (×100 magnification). Care was taken to avoid overlapping fields. Pixel size calibration was performed using a stage micrometer (160 pixels = 100 μm). The images were analyzed using the procedure described by Da Motta et al. (2003),[32] implemented with Visilog 5 (Noésis). The sludge structure was quantified by the ratio between the number of small fragments (nonfilamentous fragments with an area of less than 6.25 μm^2) and the total area occupied by the biomass on the image and filament abundance (ratio of the filament length (in pixels) to the total area occupied by the biomass on the image). Average values were calculated for about 100 images. The coefficient of variation (c.v.) for any image analysis set was less than 10% if the number of images was increased by 10%. Indeed the c.v. was between 1 and 5%, and we used 5%.

The Live/DeadBacLightTM bacterial viability stain was used according to the manufacturer's instructions (Molecular Probes). The kit provides a two-color fluorescence assay of bacterial viability based on membrane integrity using the nucleic acid stains SYTO 9 (fluorescent green) and propidium iodide (fluorescent red). When the dyes are used together, cells with intact membrane show a green fluorescence, while cells with damaged membranes show a red fluorescence. The protocol established by Lopez et al. (2005)[33] was used: 1 mL of undiluted biomass suspension was mixed with 3 μL of an equimolar mix of SYTO 9 and propidium iodide (5 μM final concentration). This short staining protocol allows direct observation of the original floc structure. Microscopic observations started 15 min after staining. Fluorescence emissions were recorded with an Airy disk confocal pinhole opening, which yielded 512 × 512 images with a 0.28 μm (x,y) pixel size (pinhole 118 μm, pixel dwell time of 3.2 μs). A constant 1 μm step size in the vertical direction was used when imaging the 3D structures. Phase-contrast microscopy was also used to visualize the structure of the flocs. The excitation maxima for SYTO9 and propidium iodide bound to DNA were 480 and 540 nm, respectively. SYTO9 was excited at 488 nm, and fluorescence emission was collected in the green channel between 505 and 550 nm. Propidium iodide was excited at 543 nm, and fluorescence emission was collected in the red channel after long-pass filter at 585 nm. The EPS of the sludge flocs was excited at 405 nm, and their autofluorescence was collected in the blue channel between 505 and 550 nm.

DNA Extraction. Every week, sludge from each reactor was sampled for molecular biological analyses. Two mL of sludge was pelleted at 15000 *g* for 10 min and then total DNA was extracted using the FastDNA spin kit for feces on the FastPrep Instrument (MP Biomedicals), according to the manufacturer's instructions. In order to concentrate bacteria from feed effluent, wastewater samples were filtered in triplicate under vacuum through sterile 47-mm membranes with a porosity of 0.45 μm (Millipore). Total DNA was extracted from the bacteria retained on the filter using a PowerWater DNA isolation kit

(MoBio Laboratories Inc.), following the manufacturer's instructions. Total DNA of all HE or UE were pooled. Before storage at −20 °C, the quality of extracted DNA was verified by electrophoresis through 0.8% (w/v) agarose gel, and its quantity was assayed in triplicate with a Nanodrop spectrophotometer (Thermo Scientific).

PCR-DGGE Experiment. PCR-DGGE was based on Laurent et al. experiment.[34] Extracted DNA from each sludge sample was analyzed by PCR using the universal bacterial primers 341F with a 40-bp GC-clamp and 518R.[35] For PCR, 1 ng·μL^{-1} template DNA was used in a final volume of 100 μL. Twenty-five microliters of each PCR product was loaded onto 8% (w/v) polyacrylamide gel (containing acrylamide and bis-acrylamide, 37.5:1) with a linear denaturant gradient ranging from 40% to 60% (urea w/v and formamide v/v). DGGE was performed at 60 °C for 15h at 100 V in 1 × TAE electrophoresis buffer with the D-code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA). Gel images were analyzed using Quantity One Quantitation Software Version 4.6.1 (Bio-Rad Laboratories).

Pyrosequencing. Pyrosequencing was used to analyze bacterial diversity. The V3 and V4 regions of the 16S rRNA encoding gene were chosen to analyze bacterial diversity, using the universal bacterial primers 339F (CTCCTACGGGAGG-CAGCAG) and 339R (TTGTGCGGGCCCCCGTCAATT) which target the V3 and V4 variable regions of the 16S rRNA gene. Pyrosequencing and PCR were conducted by the Molecular Research LB Lab (http://www.mrdnalab.com/) using standard laboratory procedures and a 454 FLX Sequencer (454 Life Sciences). The detailed methods, analysis pipeline, and results are presented in the SI.

Diversity Analyses. Rarefaction analyses were performed using PAST software (PAleontological Statistics v1.60) from http://folk.uio.no/ohammer/past/. Primer6 software (Plymouth Routines In Multivariate Ecological Research, version 6.1.6) was used to calculate the Bray−Curtis (BC) similarity index, the diversity indices (Chao1 richness and Shannon diversity indexes), and for clustering analysis coupled to two-dimensional nonmetric multidimensional scaling ordination (2D-nMDS) based on BC similarity, taking into account the presence or absence of bands and their relative intensity for the DGGE pattern.

Quantitative PCR Protocol. Quantitative PCR (qPCR) assays targeted the *intI1* gene of the class 1 RIs from total extracted DNA, using the method described by Barraud et al. (2010).[36] Quantification of the rRNA 16S encoding gene was performed in a SYBR green assay using the universal primer 338F and 518R which target the 16S rRNA gene of Eubacteria as described by Park et al. (2006).[37] Assays were performed in triplicate with a MX3005P real-time detection system (Stratagene). In order to avoid qPCR inhibitor effects, total DNA samples were serially diluted to the dilution at which quantification was not affected. For accurate quantification, the *intI*1 and 16S rRNA genes were embedded in a single standard plasmid. A full standard curve, between 10^3 and 10^8 copies of standard plasmid, was included in duplicate in each qPCR run. The total estimated bacterial cell number was obtained using quantification of the 16S rRNA encoding genes. Based on the Ribosomal RNA Database, the mean number of 16S rRNA encoding genes per bacterial cell is currently estimated to be 4.1.[38] Therefore, 16S rRNA encoding gene copy numbers were divided by 4.1 to estimate the total bacterial load. The relative abundance of class 1 RIs was calculated by dividing their

concentration by the estimated bacterial load. For all statistical tests, the Mann−Whitney was implemented with StatView 5.0 software (SAS institute Inc.), and p values <0.05 were considered significant.

■ RESULTS

General Performance of the CAS System under Different Feeding Procedures. The overall performance of the two CAS reactors was measured by classical physicochemical parameters until steady-state was reached. The purification performance of the HE reactor was equivalent to the UE reactor; for both reactors TSS, COD, and TN removal rates averaged greater than 95%, 90%, and 70%, respectively, and the SVIs decreased to 50 mL·g^{-1} of TSS after 16 days of operation. Specific differences in the HE reactor were observed in a significant increase in the organic fraction of the sludge from 0.8 ± 0.02 to 1 ± 0.03 VSS·TSS^{-1} from day 22 to the end of the experiment (Figure S2−B) and a significant increase in the soluble COD content of the treated HE from 22 ± 1.8 to 57 ± 4.6 mg·L^{-1} by the 34th day (Figure S2−C).

Evolution of the Sludge Structure. The sludge structure and morphology were examined using optical microscopy combined with images analysis. Throughout the first 26 days, no difference in activated sludge floc morphology was observed between the two reactors. At the end of the experiment, phase contrast microscopy showed the presence of a greater proportion of floc fragments (i.e., very small flocs, see Figure 1C) in the HE reactor than in the UE reactor (0.023 vs 0.008 small fragments per μm^2, respectively) (Figure 1A).
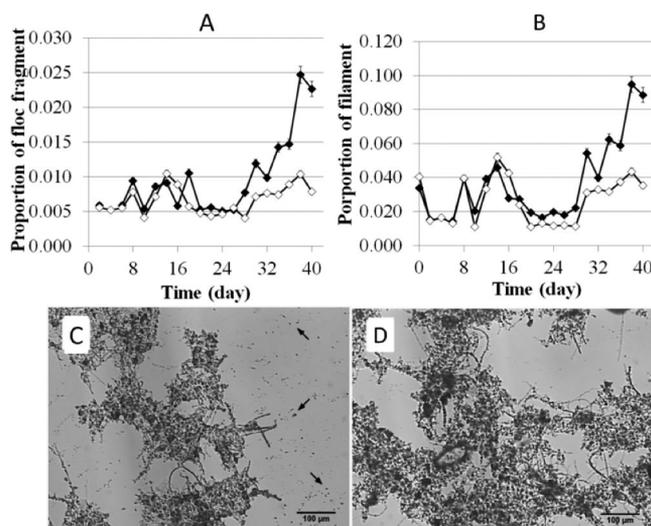
Figure 1. (A) Ratio of fragments of flocs (number of small fragments/total floc area) and (B) ratio of filaments (filament length/total floc area) over time in the HE (◆) and UE (◇) reactor. Examples of phase contrast micrograph of activated sludge floc morphology at the end of the experiments in the HE (C) and the UE feed reactor (D). The arrows indicate fragments of floc in the activated sludge of the HE reactor.

At the end of the experiment, a high density of live filamentous bacteria as well as damaged bacteria which were principally nonfilamentous, was observed with CLSM in the flocs from the HE reactor (Figure 2B vs 2E). This difference was confirmed by quantitative image analysis where, after 26 days of experiment, the ratio of filaments per μm^2 increased
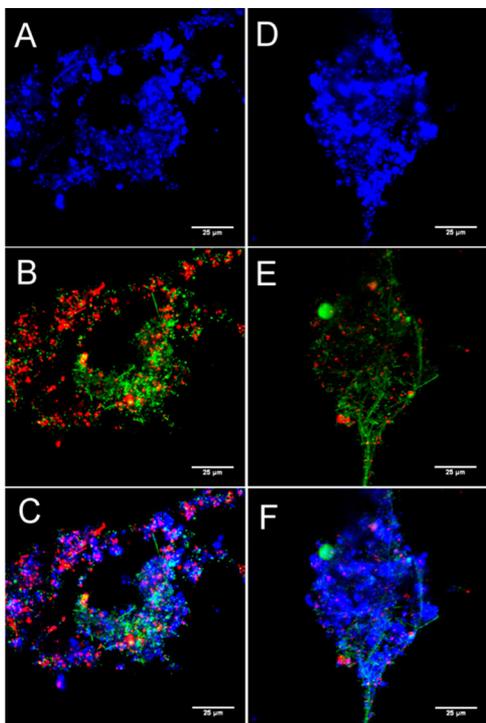
Figure 2. CLSM images of activated sludge at t = 40 days in the UE reactor showing (A) autofluorescence; (B) viability staining; (C) merge of autofluorescence and viability staining; and in the HE reactor showing (D) autofluorescence; (E) viability staining; (F) merge of autofluorescence and viability staining. The green and red fluorescences correspond to living and damaged bacteria, respectively. The blue fluorescence corresponds to the fluorescence of EPS.[69]

genes was used to study the influence of HE on the bacterial diversity in sludge.

Average observed bacterial concentrations in the two reactors were similar and stable over time, at respective $1.82 \times 10^{11} \pm 6.69 \times 10^{10}$ and $3.58 \times 10^{11} \pm 3.44 \times 10^{10}$ estimated bacteria·L^{-1}, except for the first week when the concentration in the HE reactor was significantly higher (Figure S3). Bacterial loading in the HE and UE was relatively constant throughout the experiment, albeit higher for HE than UE ($3.0 \times 10^{11} \pm 2.0 \times 10^{11}$ and $1.2 \times 10^{11} \pm 6.1 \times 10^{10}$ of estimated bacteria cells·L^{-1}, respectively).

A total of 49 different bands were noted on the PCR-DGGE profile (Figure S4). The band patterns were similar to each other, with a minimum BC similarity index of 91.1%. Therefore, in terms of bacterial diversity, feeding HE or UE did not affect the main bacterial taxa of the sludge community. Assuming that PCR efficiencies were identical for all targeted sequences, the relative intensity of the bands (semiquantitative analysis) was used to follow the evolution of the bacterial communities (Figure 3A).

This semiquantitative analysis showed that the bacterial community of the HE reactor changed over time. In the first step, the bacterial community evolved until the third week (T3), which corresponded to the point at which its structure was most different from its initial structure (BC similarity index between T0 and T3 = 63.3%). At T4, T5, and TF (end of the experiment) bacterial community shared a BC similarity index higher than 89.9%, indicating that the bacterial community had reached equilibrium. By comparison, the same semiquantitative analysis showed that the bacterial community in the UE reactor did not evolve (BC similarity index consistently higher than 84%). Overall, the semiquantitative analysis showed that while no appearance or disappearance of main bacterial taxa occurred over time in the HE reactor after steady-state was reached, an early shift of the balance within the community occurred in the first weeks following the start of the experiment.

In order to further understand this evolution, bacterial communities from both reactors were investigated by pyrosequencing of a portion of the 16S rRNA encoding gene. The diversity analysis comprising of the rarefaction curves, the

from 0.018 to 0.089 in the HE reactor (Figure 1B). The EPS of the sludge was denser in the floc from the HE reactor (blue fluorescence on the Figure 2A vs 2D), suggesting a higher rate of EPS secretion by bacterial biomass of the sludge.

Evolution of Bacterial Communities. PCR-DGGE coupled with pyrosequencing of the 16S rRNA encoding
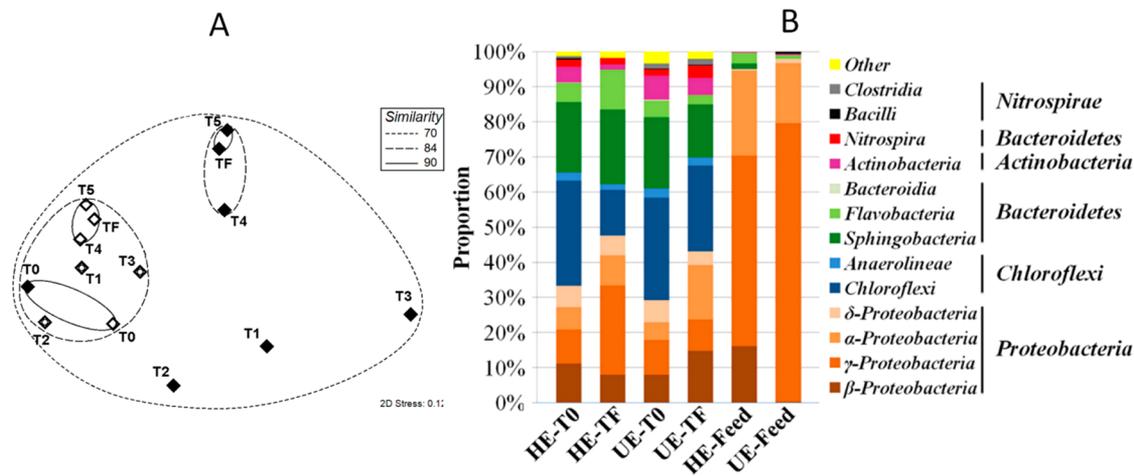
Figure 3. (A) 2D-nMDS map based on the semiquantitative analysis of the DGGE profiles showing the evolution of the activated sludge bacterial community in both reactors (◇ UE, ◆ HE). This 2D projection of the BC similarity matrix allowed visualization of the similarity between each bacterial community over time, i.e. the distance between diamonds. Plain and dashed lines represent the differing percentage of similarities. (B) Proportion of bacteria classes and phyla recovered from the HE and UE feed to the reactors (HE-Feed and UE-Feed) and from the HE and UE reactor sludges at the beginning (HE-T0 and UE-T0) and the end (HE-TF and UE-TF) of the experiment.

bacterial diversity indices (Chao1 richness and Shannon indices), and their genus affiliation are given in the SI. Regarding the bacterial genera present in the HE reactor at the initial (T0) and final (TF) time of the experiment, 85 genera among the 121 genera present at the beginning of the experiment were still detected among the 118 genera present at the end. These 85 genera represented 93 to 94% of the total bacterial population, confirming the interpretation of the similar PCR-DGGE band pattern that HE did not majorly disturb the bacterial community within the reactor. The evolution observed within the HE reactor was thus mainly due to evolution of the proportion of the bacteria already present.

The proportions of each bacterial class and phylum are shown in Figure 3B. Between the T0 and TF a major reduction (−16.9%) and augmentation (+15.7%) was observed in the bacterial classes *Chloroflexi* and *γ-Proteobacteria*, respectively, in the HE reactor (HE-AS-T0 vs HE-AS-TF in Figure 3B).

The analysis of the variations within the bacterial genera between the beginning and the end of the experiment showed major variation (>1%) of 10 bacterial genera in the HE reactor (Figure 4). The most noticeable examples of this phenomenon
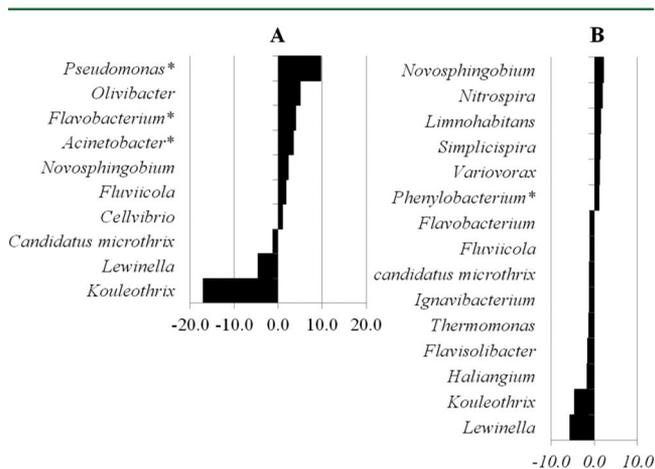
Figure 4. Major positive or negative variations (in excess of 1%) of the proportion of bacterial genus in (A) the HE reactor and (B) the UE reactor between the beginning and the end of the experiment. * corresponds to genus found also in the effluents used for the feed of the reactors.

were observed in the following: (*i*) the filamentous bacteria of the *Koulethrix* genus belonging to the *Chloroflexi* class, which are the most abundant bacteria in the sludge (Figure 3B), reduced by 17.1% and (*ii*) the floc-forming bacteria of the *Pseudomonas* genus belonging to the *γ-Proteobacteria* (Figure 3B) increased by 9.7%. In contrast, the variations observed in the UE reactor were lower (Figure 3B, Figure 4).

Although the main evolution of the bacterial community was due to a shift in the bacterial balance of main taxa already present, the in-depth analysis using pyrosequencing revealed the introduction of some new bacterial taxa by the HE into the sludge of the reactor. The analysis of all operational taxonomic units (OTUs) which were not initially detected in the HE reactor at the beginning of the experiment, but which were detected in the HE itself and in the HE reactor at the end of the experiment (Table 1), showed that the HE specifically introduced 24 OTUs in the sludge of the HE reactor. Among these OTUs, 5 and 6 respectively belonged to the *Acinetobacter*

Table 1. Proportion (in %) of OTU, Defined for a 3% Sequence Identity Cutoff, and Their Affiliated Genus, Introduced by HE into the Activated Sludge

affiliated genus	number of OTU	proportion in HE	proportion in HE reactor at TF
Achromobacter	1	0.2	0.1
Acinetobacter	5	4.8	0.8
Agrobacterium	1	0.3	0.1
Comamonas	3	10.8	0.3
Diaphorobacter	1	0.4	0.1
Flavobacterium	2	0.1	0.7
Ochrobactrum	1	1.4	0.1
Phenylobacterium	1	0.2	0.1
Pseudomonas	6	3.1	7.3
Shinella	1	0.4	0.4
Sphingobacterium	1	1.6	0.1
Stenotrophomonas	1	0.8	0.1

and *Pseudomonas* genera. A large increase (+7.3%) of *Pseudomonas* proportion in the total bacterial community was observed between T0 and TF. For the UE reactor, the same analysis revealed that only 5 genera were introduced by the UE (*Afipia, Ochrobactrum, Paracoccus, Pseudomonas, Shinella*). Moreover their proportion in the bacterial community at the end of the experiment did not exceed 0.1% (data not shown).

Class 1 Resistant Integron (RI) Evolution. Class 1 RIs relative abundances were assessed as an indicator of antibiotic resistance of the sludge bacterial communities. The relative abundance of class 1 RIs in the HE was higher than that in the UE (0.230 (±0.120) vs 0.030 (±0.028) of class 1 RIs per estimated bacterial cell). HE feeding resulted in an increase of class 1 RIs relative abundance in the sludge during the first weeks of the experiment (Figure 5).
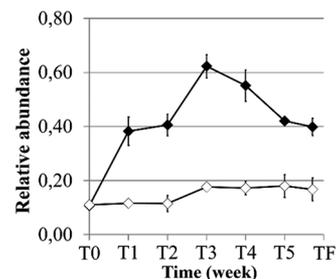
Figure 5. Evolution of the relative abundance of class 1 RIs over time in the HE (◆) and UE (◇) reactors.

By comparison with the UE reactor, the relative abundance of class 1 RIs in the HE reactor increased up to 3.5-fold by the third week of the experiment (T3), the time when the bacterial community was the most divergent from its initial state (Figure 3A). Then the relative abundance decreased from 0.62 ± *0.04* to 0.42 ± *0.003* class 1 RIs per estimated bacterial cell until the fifth week (T5), i.e. the time corresponding to the equilibrium of the bacterial community. Nevertheless, the relative abundance of class 1 RIs was still significantly different at TF. In the UE reactor the initial relative abundance of 0.11 ± *0.01* of class 1 RIs per estimated bacterial cell did not vary although the UE influent relative abundance was lower (0.030 (±*0.028*) of class 1 RI per estimated bacterial cell).

■ DISCUSSION

HEs are characterized by high concentrations of surfactants, pharmaceuticals, potentially hazardous bacteria, and ARD.[39] Thereby, they can influence biological wastewater treatment in terms of (i) overall activity, (ii) floc structure, (iii) bacterial diversity, and (iv) ARD dissemination. In order to obtain an accurate characterization of this influence, a multiscale approach, coupling different types on analysis, was carried out during the monitoring of two identical lab-scale CAS reactors treating HE and UE.

Previous studies have reported the stability of performance of biological processes fed with synthetic mixtures of antibiotics or real HE.[40,41] In accordance with their observations, the initial CAS ecosystem studied here was quickly able to adapt its activity to the specific makeup of HE in terms of suspended solids and organic matter content. However, a more in-depth investigation uncovered impacts of the HE on the organic content of sludge and on the soluble COD content of the treated effluent.

HE Induced Floc Erosion. Acclimatization of the active biomass, probably occurring either at the floc structure level (i.e., defloculation) or at the bacterial community level, could explain these observations. Similar amplified effects have been mentioned by other authors who observed floc disintegration and bacterial lyses after application of high concentrations of a single antibiotic in batch condition.[29,42−44] This suggests that the HE did not induce any acute toxicity on the biomass but altered the physical structure of flocs.

It has been shown that some surfactants and pharmaceuticals found in HE result in a decrease in the mean surface of sludge flocs.[43,45,46] In our study, assessment of the proportion of filaments indicated either a filamentous bulking phenomena also described elsewhere[47] or a floc erosion/disruption leading to the release of core filaments. In our study, the analysis of bacterial diversity by pyrosequencing did not support the filamentous bulking hypothesis, as it revealed a significant reduction in the proportion of filamentous bacteria belonging to the *Chloroflexi* class, represented mainly by *Kouleothrix* spp., the most abundant bacterial genus in the sludge. *Chloroflexi* are widely distributed in activated sludge and are chiefly located in the core of flocs, probably explaining their involvement in the stabilization of the floc backbone.[48,49] All our observations tended to show that HE leads to the erosion of sludge flocs, resulting in an increase in floc fragments, exposure of 'core filaments', and the degradation of the organic load of the treated effluent.

HE Promoted Excretion of EPS. Toxic compounds, at nonlethal concentrations, induce reactions in the flocs, biochemically characterized by production of EPS.[50,51] Such EPS excretion has been observed during exposure of flocs to cyclophosphamide.[52] In our survey, such molecules were found in the HE but were not detected in the UE. Indeed, average concentrations of the cytotoxic agents cyclophosphamide and ifosfamide were, respectively, 290 ± 159 ng·L^{-1} and 265 ± 271 ng·L^{-1}, for the anesthetic lidocaine, 537 ± 560 ng·L^{-1}, and for the antibacterials ciprofloxacin and sulfamethoxazole average concentrations of 455 ± 172 ng·L^{-1} and 280 ± 70 ng·L^{-1}, respectively, were quantified (Table S2). Denser floc matrices, observed by autofluorescence coupled with bacterial staining in the HE feed, suggested that during HE treatment EPS content of the flocs increased. It seems reasonable to infer that HE promoted EPS production by the biomass. This hypothesis is supported also by the observed increase in the proportion of important floc-forming bacteria in the HE reactor: *Flavobacteria* spp. and *Pseudomonas* spp. (Figure 4), which are known to form biofilms under stress- or in wastewater-conditions.[12,53]

HE Modified Bacterial Community. While clear morphological shifts of flocs were observed in sludge fed with HE, the diversity of the initial bacterial communities in the sludge was not strongly impacted. Nevertheless, a bacterial balance shift was observed in the HE reactor before the morphological shift of the flocs. Indeed this fast adaptive response of the bacterial community, probably due to its flexibility, explained the consistent performance of the purification process throughout the experiment. Other studies reported that the adaptation of the bacterial community is more likely related to the flexibility of the population than its diversity.[54,55] Specifically for pharmaceutical wastewaters, Lapara et al. (2002)[56] showed that the flexibility of the initial bacterial community, in response to influent variations allowed for consistent process performances. Another study[31] using synthetic wastewater spiked with pharmaceuticals at concentrations above 50 μg·L^{-1} described a minor but consistent structural divergence of the bacterial community in a lab scale CAS reactor and also a reduction of the bacterial diversity, while other reactor parameters, such as the nitrification rate, were not affected. In our study, the use of real HE tended to exhibit a similar evolution of the bacterial community, except that reduction in bacterial diversity was not observed.

HE Induced Increase of Class 1 RI. The occurrence of ARD in HE is considered as an efficient way of spreading antibiotic resistance in WWTPs.[57,58] Previous studies based on cultivable approaches pointed out the occurrence of some hazardous bacteria, including antibiotic resistant strains (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* spp.) in WWTPs treating HE.[21,57−59] The objectives of our study was to obtain a global assessment of the antibiotic resistance dissemination in a WWTP operating HE. We thus focused on a culture-independent method using the relative abundance of class 1 RIs as a genetic marker of ARD. We showed that HE feeding increased the relative abundance of class 1 RIs in the bacterial population of the sludge, thus leading to an increased potential for ARD dissemination. The coevolution of the relative abundance of class 1 RIs and the bacterial community in the HE reactor suggests that this behavior could be due to the introduction or the *in situ* development of bacteria that possess these genetic elements, rather than horizontal gene transfers. This hypothesis is strongly supported by the appearance within the sludge after HE feeding of *Pseudomonas* and *Acinetobacter* genera, which are known as important RI carriers.[60]

Pseudomonas spp. are ubiquitous environmental bacteria. Their high adaptation capacities seem to be linked to their genome plasticity, which is composed of a multitude of exogenic acquired DNA.[61] Moreover, *Pseudomonas* spp. strains are characterized by their capacity to form the following: (i) biofilms in medical infections, increasing their resistance to antibiotic treatment,[62] and (ii) flocs in WWTP, leading to their development in activated sludge ecosystems.[63] This bacterial genus is also known for its ability to metabolize a wide diversity of toxic compounds such as insecticides, antiseptics, herbicides, and surfactants and to be typically recovered from environments exposed to disinfectant or antibiotic pressures.[64] In this study, the HE feeding led to a transient state of the bacterial community before it reached its equilibrium. During this

transient state ecological niches could have been created, and *Pseudomonas* spp., probably more adapted to the conditions shaped by the HE in the HE reactor, could have had a selective advantage to grow in the bacterial community. This assumption is supported by a survey[64] which demonstrated, by an equivalent comparative metagenomic approach, that the contamination of river sediments by quaternary ammonium compounds, substances typically found in HE, promoted the enrichment of bacterial communities in *Pseudomonas* spp.

Stress conditions increase the diversity of genetic variants of *Pseudomonas* spp., leading to their adaptation to changing conditions and to the emergence of antibiotic-resistant strains.[65] Moreover these types of bacteria have been shown to be both acceptors and donors of broad host range plasmids in sludge.[66,67] We conclude that the introduction of *Pseudomonas* strains originated from HE in sludge could increase the risk of antibiotic resistance dissemination in WWTP processing HE. Furthermore, stress conditions, including antibiotics, also induce the acquisition of antibiotic resistance gene cassettes by RIs *via* the SOS response.[68] HE offer ideal conditions to promote genetic evolution in bacterial communities.

Environmental Relevance of the Study. This work showed that the specific treatment of medical care effluents did not unduly disturb the activated sludge process, while it did however affect the floc structure, the production of the floc EPS matrix, and the bacterial population balance. It did also ultimately alter sedimentation and the treated water quality.

On the other hand, HE increased the ARD in the sludge, *via* class 1 RIs, and promoted the introduction and the development of *Pseudomonas* ssp. In order to deeper understand ARD introduced by HE in sludge biomass, accurate characterization of the resistome embedded by bacterial communities present in HE treatment should be considered in future studies.

Indeed, more attention should be paid toward downstream treatments and land-application of sludges produced by WWTPs treating HE effluents. The work by Burch et al. team[70] on the removal of some ARD in the sludge treatment processes highlights the importance of process technology and parameters choice (i.e., aerobic/anaerobic, temperature), which in some cases could increase or decrease the proportion of class 1 RI containing bacteria in treated sludge. Moreover, in the treatment of HE, membrane bioreactors using ultrafiltration membranes have been reported to be efficient process options to avoid sedimentation problems of the biomass, to increase the treated effluent quality, and specifically to remove class 1 RI from HE (www.pills-project.eu).

■ ASSOCIATED CONTENT

ⓈSupporting Information

A full description of the sampling sites, the main physico-chemicals methods and characteristics of the pharmaceuticals compounds load within the effluents, and the CAS reactor used as well as the methods used for the standard generation for real-time PCR, the pyrosequencing of the 16S rRNA encoding genes and all supporting figures and tables provided in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Comparaison du traitement d'effluents hospitaliers par boue activée et réacteur membranaire : efficacité et impacts.

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Dans le cadre de l'amélioration des procédés de traitement d'effluents hospitaliers, cet article propose la comparaison d'un système de traitement biologique membranaire (BAM) avec un système à boue activée traditionnel (BA) pour le traitement d'un effluent hospitalier.

Le fonctionnement de 2 pilotes (BA et BAM) a été suivi pendant 60 jours et leurs performances ont été comparées, avec un suivi de l'évolution de la qualité biochimique des flocs par microscopie confocale avec marquage fluorescent. La spectrométrie de fluorescence 3D a été introduite comme technique susceptible de permettre le suivi de la qualité des influent et des effluent au cours du temps.

Les deux systèmes de traitement aboutissent à des performances épuratoires équivalentes sur les paramètres classiques de pollution, le BAM ayant cependant des capacités à la rétention des MES supérieures (96,1% contre 89,1%). Un panel de substances pharmaceutiques a été mesuré en amont et en aval des deux procédés. Les résultats, comparés à ceux de la littérature, dans des conditions de gestion du procédé pouvant cependant être différente, montrent, de manière générale, de meilleures performances pour le BAM que pour le BA. Comme cela est retrouvé dans la littérature, certaines molécules ne sont éliminées de manière très partielle par l'un et l'autre des procédés. Par exemple, le paracétamol, le kétoprofène, l'ibuprofène, la caféine, le métronidazole, le bézafibrate, le fénofibrate, la pravastatine, le ramipril, le propanolol, l'aténolol, le sotalol, la carbamazépine, le losartan, l'oxazépam et isosfamide sont modérément éliminés par BAM, mais leur élimination par le BA était beaucoup plus faible. Aucune élimination a été réalisée pour la roxithromycine et reste faible pour le triméthoprime (31,4%) et le sulfaméthoxazole (50%).

Le mode d'élimination des composés pharmaceutiques pouvant être lié à deux modes principaux, la biodégradation et/ou la sorption, les constantes biologiques Kbio et de sorption ont été mis en parallèle des taux d'élimination dans les 2 types de réacteurs, pouvant permettre d'avancer des hypothèses quant à la prédominance d'un

des mécanismes dans le procédé (comme pour le sulfametoxasol par exemple)

Le suivi de l'évolution des EPS, par analyse biochimique, par microscopie confocale et par spectrométrie de fluorescence, a, montrée que les effluents hospitaliers modifiaient la structure et la composition des flocs. Le BAM favoriserait une production de EPS (et notamment des protéines) plus importante que le BA, pouvant être liée à un âge des boues plus important. Cela à des conséquences sur le colmatage des membranes.

Performances and effects on the EPS of submerged membrane bioreactor (MBR) compared to conventional activated sludge treatment for hospital effluent treatment

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Abstract

Performance of a submerged membrane bioreactor (MBR) and conventional activated sludge (CAS) for the treatment of hospital wastewater was investigated. Two pilot-scales were designed and operated for more than 60 days. Lab scale experiments were conducted and microscopic techniques used to monitor and characterize the extracellular polymeric substances of sludge (EPS) as an indicator of biochemical modification of bacterial community under stress. The removal of the organics compounds by MBR was more effective than with the CAS. After 15 days of exposure to the hospital effluent, a significant increase of soluble EPS concentration in MBR was detected, certainly due to the occurrence of pharmaceuticals or to more or less toxic molecules in the effluent. Paracetamol, ketoprofen, ibuprofen, caffeine, metronidazole, bezafibrate, fenofibrate, pravastatin, ramipril, propanolol, atenolol, sotalol, carbamazepine, losartan, oxazepam and isosfamide were moderately removed by MBR, while their removal by CAS was much lower. The monitoring of EPS concentrations, estimated by biochemical analysis, confocal microscopy and fluorescence spectrometry, showed the impact of the process on production the EPS.

Key words: Membrane bioreactor, EPS, Pharmaceuticals, Conventional activated sludge

1. Introduction

In recent years, increasing attention has been carried out to the presence of emerging pollutants in wastewater, in surface water and in groundwater (Daughton and Ternes, 1999; Heberer, 2002; Barceló, 2003; Daughton, 2004; Petrovic et al., 2009). These pollutants include surfactants, pharmaceuticals and personal care products (PPCPs), endocrine disruptors, illicit drugs, gasoline additives and many other groups of compounds. In most

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cases, there are not yet all listed in the water quality regulations but may be candidates in the future, at least under surveillance list, depending on research on their potential health and/or environmental effects and the results of monitoring of their occurrence.

At the urban scale, effluents discharged by hospitals represent about 20% of the total organic PPCPs load found in the urban sewer (Carballa et al., 2004) and are considered as an important local source of these compounds: a great variety of micro-contaminants resulting from diagnostic, laboratory and research activities on one side and medicine excretion by patients on the other. (Kummerer, 2001; Petrovic et al., 2003; Carballa et al., 2004; Onesios et al., 2009).

Some researches studied on the *on-site* treatment of hospital effluent before the release into the sewage network (Gautam et al., 2007; Lenz et al., 2007; Pauwels and Verstraete, 2006; Interreg PILLS project, 2013). Recently studies have assessed the risk of hospital effluent on an ecotoxicological point of view. But if they concluded that the dilution in urban sewage reduced the acute toxicity of the total effluent, the real effects of emerging pollutants accumulation on environment are controverted (Jean et al., 2012).

The conventional activated sludge CAS is currently the principal barrier currently used to decrease the release of harmful contaminants into the environment and to slow down the risk induced by pharmaceutical residues, if the treatment facilities and waste disposal in wastewater treatment plants (WWTPs) are designed, operated and managed properly (Ghosh et al., 2009, Choubert et al., 2009). Further understanding of the effectiveness of conventional WWTPs and new wastewater treatment technologies for removing pharmaceutical residues will play an important role in addressing these issues (Ghosh et al., 2009). We consider that about 50 % of the PPCPs load could be retain a conventional biological WWTP but depending of a lot of parameters, like molecule properties, type of water process and treatment management (Martin, 2012). The main mechanisms responsible for the pharmaceutical compounds removal from water are sludge sorption, biodegradation by microorganisms and, in the MBR system, retention by the membrane (José et al., 2010). Consequently, variable discharged quantities of pharmaceuticals can reach surface and ground waters (Farre´et al., 2001). (Carballa *et al.,* 2004) indicated that although during the primary treatment process, pharmaceuticals compounds were not removed efficiently (ranged from 20 to 50%), the aerobic activated sludge process caused a significant reduction in all compounds detected, between 30 and 75%, with exception for iopromide, which remained in the aqueous phase. (Joss et al., 2005) did not observed an evident correlation between the chemical structure of compound and biological removal efficiency, which varied strongly from

compound to compound because of the mechanism of elimination from the aqueous phase (Barrat et al., 2010).

Processes with membrane, such membrane bioreactors (MBR) or micro/ultra filtration as a tertiary treatment, have been set up to improve the efficiency of PPCP removal. MBRs usually operate at high sludge retention times and high concentrations of biomass, allowing an intensification of biological processes by the implementation of resistant and low-growth biomass (DeWever et al., 2007; Delgado et al., 2009). These conditions may increase the elimination of contaminants with special characteristics like pharmaceuticals compounds, such as low bio-degradability and low concentration (by co-metabolism phenomena),. (Clara et al., 2005) found that diclofenac removal failed by size exclusion, but a partial removal could be obtained by rising the sludge retention time. Ibuprofen on the other hand was removed to a high degree (> 90%). In contrast to (Clara et al., 2005 a), a study by (Radjenovic et al., 2007) indicated a better pharmaceutical removal with MBR compared to CAS for, as a example, 87.4% compared to 50,1%, 58.7% compared to 0%, and 71,8% compared to 27,7% for diclofenac, metoprolol and clofibric acid respectively.

Nevertheless, the main problem in membrane application is a rapid decline in the permeation flux due to membrane fouling, which requires frequent membrane cleaning/replacement, thus increasing the running costs (Judd et al., 2004). Many studies indicate that the soluble EPS play a major role in fouling (Rosenberger et al., 2002; Rosenberger et al., 2006). Some authors attributed primarily it to proteins, present in the effluent or produce by the microorganisms (Hernandez et al., 2005; Meng et al., 2006), but a larger number of recent publications indicates that soluble polysaccharide is also one of the main molecules affecting MBR fouling (Le-Clech et al., 2006; Rosenberger et al., 2005; Lesjean et al., 2005; Nataraj et al., 2008; Alrhmoun et al., 2015). This last point still needs examination in the case of the presence of toxic compounds, especially on the understanding of the sludge development and subsequent characteristics, because it is known that these compounds could induce the production of EPS.

The aim of this paper is to compare the CAS and MBR performances in treating hospital wastewater at pilot scale. In addition, this work investigated the effects of presence the toxics agents on the EPS production.

2. Materials and Methods

2.1. Study area

The hospital effluent (HE) samples used in this study were collected from the sewerage system of the clinical activities of the Limoges hospital (France). Average characteristics of

wastewater and activated sludge (CAS) used as inoculums during the experiments are detailed in table 1.

Table1

Physicochemical characteristics of the hospital effluents (HE) and activated sludge (AS).

	COD (mg/L)		N (mg/L)		TSS(g/L)	TVS(g/L)
	Total	Soluble	Total	Soluble		
HE	412.5±5	173.5±5	128.9±4	95±4	0.199	0.091
AS	1201±5	285±5	-	-	6.214	1.35

2.2. Reactors and operating conditions

Two lab-scale pilots have been used: a conventional activated sludge system (CAS) and a membrane bioreactors (MBR), with, if possible, identical operating conditions (Table 2)

Table2

Key operational parameters of CAS and MBR systems investigated.

Parameter	CAS	MBR
HRT (h)	15,3	15-24
SRT (days)	15	15-20
Flow (m3/h)	0,0009	0,0018
Temperature (C°)	17-20	16-19
PH	7,0-8,0	7,0 - 8,0
Dissolved Oxygen (mg/L)	2,0-4,5	2,0 - 4,5
Aerobic tank (L)	14	30

2.2.1. Conventional activated sludge system (CAS)

The CAS (Fig.1. A) had a total volume of 14 L and was continuously fed with wastewater collected each 7 days at the hospital. Wastewater was kept at 4°C in an agitated tank where it was directly pumped to feed the pilot. Influent flow rate was 21.6 L.d^{-1} corresponding to a hydraulic residence time of 15.3 h^{-1} in the aeration tank. Aeration was operated by repeated aerobic/anoxic cycles (3h/3h) in order to ensure nitrification and denitrification. Air flow rate was adjusted daily to maintain a dissolved oxygen concentration between 2 and 4 mg.L^{-1} in the reactors during aerated phases and avoid oxidation limitation by oxygen concentration. Solids residence time (SRT) was maintained at 15 days throughout the experiments: settled sludge was wasted every two day accordingly considering sludge losses through the discharged effluent. The experiment was conducted over a period of 60 days.

A

B
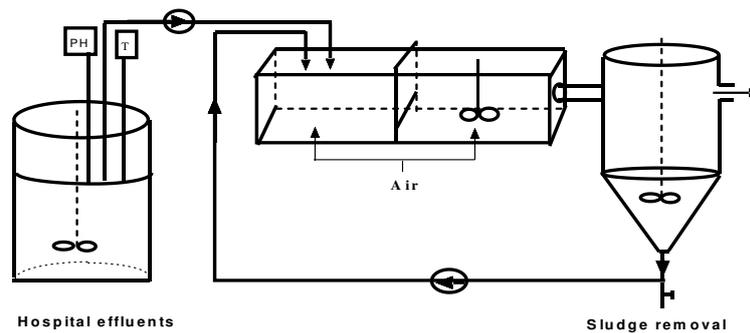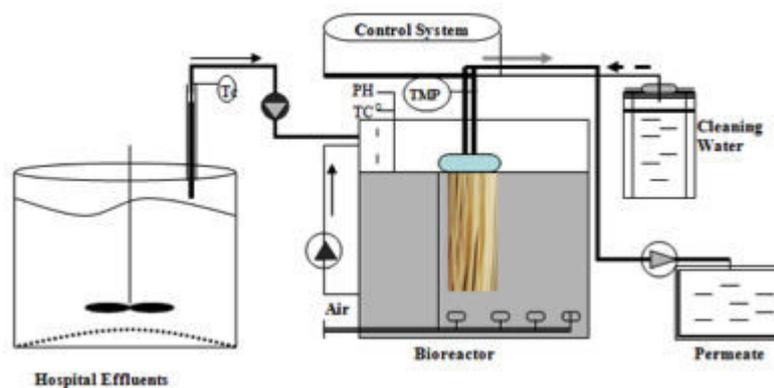
Fig. 1. Shematic diagrams of CAS (A) and MBR (B) systems treatment.

2.2.2. Membrane bioreactor (MBR)

The Membrane bioreactor (polymem – Toulouse- France - Fig. 1. B) was constituted of a 30 1 bioreactor and ultrafiltration-shaped hollow fibre membrane module immersed in the bioreactor. Hollow fibres were made of polypropylene with a pore size of 0.05 μm. Aeration was done through diffusers at the bottom of the reactor to provide oxygen for biomass growth as well as shear to reduce cake formation at membrane surface. Dissolved oxygen levels were maintained between 2 and 4.5 mgO$_2$/L. The membrane permeate was continuously removed by a peristaltic pump under a constant flux (1.8 L/h), and the trans-membrane pressure (TMP) constantly measured to monitor the extent of membrane fouling. The operation was stopped when the TMP reached 26 kPa to maintain the flux at a constant value. The hydraulic retention time (HRT) was ranged from 15 to 24 h, the temperature from 17 to 20°C and pH from 7 to 8.

2.3. Analytical methods

The physico-chemical characteristic of wastewaters and sludge were determined every two days. The Chemical Oxygen Demand (COD) and Total Nitrogen (TN) were carried out on both total and soluble fraction after samples filtration at 1.2µm. COD was measured by the closed reflux colorimetric method (ISO 15705:2002), and TN was assessed using the alkaline persulfate digestion with colorimetric method (Hach company) (HCT 191, ISO 15705 and HACH DR/2000). Measurements of total and volatile suspended solids (TSS and VSS) were done according to the normalized method (AFNOR, NF T 90-105).

2.4. Analysis of pharmaceuticals

Pharmaceuticals analyses in the wastewater samples were performed by IANESCO laboratory (Poitiers, France). Water samples were enriched by liquid-solid phase (SPE) by using Osis HLB cartridges (6ml, 200mg) from waters. The SPE extracts were injected in liquid chromatography- mass spectrometry (LC-MS/MS) applying electrospray ionization (ESI) under high-resolution MS conditions. Acquisition was performed in selected reaction monitoring (SRM) mode and two transitions (quantification, confirmation) were obtained for each compound. Quality control (QC) was assured by measuring two transitions for each analyse and each internal standard, comparing retention time of analyse with the retention time of the internal standard in each sample, duplicates, numerous blanks, and QC standards. The global analytical error was ± 0.75µg/L.

2.5. Analysis of total protein, humic substances and polysaccharides

Protein content, expressed in mg equivalent of bovine serum albumin per gram of VSS for the soluble polymer, was determined according to the method of Lowry et al. (1951) with a correction for the humic-like substances. Humic-like substances were measured with the Folin-Ciocalteau phenol reagent in the same trial as the protein by omitting the $CuSO_4$. Results were expressed in mg equivalent of humic acid per gram of VSS for the soluble polymer. Polysaccharides were determined according to the method of Dubois et al. (1956) and the results expressed in mg equivalent of glucose per gram of VSS for the soluble polymer.

2.6. Confocal laser scanning microscopy

EPS was measured by confocal laser scanning microscopy and pictures was statically analysed by Image J software according (Alrhmoun at al., 2014). PS and PN staining was carried out according to the modified procedure of Chen et al., (2007). Bio samples were centrifuged to remove supernatant, washed twice with 1× phosphate-buffered saline (PBS)

buffer (pH 7.2) and kept fully hydrated in 2 mL centrifuge tubes covered with aluminium foil. For PS staining, 100 µL of concanavalin A conjugated with tetra -methylrhodamine (Con A, 250 mg L^{-1}, Molecular Probes, and Carlsbad, CA, USA) was first dropwise to the sample and incubated for 30 min to stain α-mannopyranosyl and α glucopyranosyl sugar residues. For PN staining, 100 µL of sodium bicarbonate buffer (0.1 M) was introduced to the sample to maintain the amine groups in non-protonated form. Subsequently, 100 µL of fluorescein isothiocyanate solution (FITC, 1 g L^{-1}, Fluka) was supplemented and incubated for 1 h to bind to proteins. Samples were washed tow times with 1× PBS buffer after each staining stage to remove loosely bound and excess dyes. Finally, sectioned granule or biofloc samples were mounted onto microscopic glass slides for observation of the distribution of PS and PN by a confocal laser scanning microscopy equipped with an Ar–He–Ne laser unit and three barrier filters. The image acquisition settings, such as laser intensity, numerical aperture, gain and offset settings were adjusted according to Toh et al., (2003) and the levels were kept constant through observation.

SYTO® 9 BacLightTM bacterial stains was used according to the manufacturer's instructions (Molecular Probes, Eugene, Oregon, USA). The kit provides a three-color fluorescence assay of bacterial relying on membrane integrity: viable bacteria are stained by SYTO® 9 and fluorescein green, while damaged bacteria are stained by propidium iodide and fluoresce in red. Protocol established by (Lopez et al., 2005; Baker A et al., 2004) was performed: 1 mL of undiluted biomass suspension was mixed with 3 L of a mixture of equal parts of SYTO® 9 and propidium iodide. This short staining protocol allowed direct observation of the original floc structure and the time-lapse microscopy. No centrifugation or fixation steps were needed. Microscopic observations started 15 min after staining. Excitation maxima for SYTO® 9 and propidium iodide bound to DNA are 480 and 540 nm, respectively (Reynolds D M et al., 2002).

To capture the image series, a Leica TCS LSI-AOTF confocal microscope (Leica Microsys- tems, Germany) equipped with 488 and 532 nm laser diode was used with an HCX 5×0.5. The bandwidth of the detected fluorescence wavelengths has been optimized to uniquely channel the maximum emission in sequential mode to avoid potential cross-talking (502–530 nm for SYTO® 9 and 600–630 nm for propidium iodide). Fluorescence emissions were recorded within 1 Airy disk confocal pinhole opening and 1024 × 1024 images at a 1.36 µm (x,y) pixel size were obtained. Instead of selecting a constant step size in the vertical direction, the step size was determined by choosing start and end points in the z-direction of

the flocs, and by then selecting a number of optical sections. The resulting voxel depths for the flocs analyzed ranged from 1 to 2 mm.

2.7. Spectroscopic analysis

Spectroscopic analysis was conducted by applying 1) ultraviolet (UV)-visible spectroscopy (Pharma Spec 1700,Shimadzu Corp., Kyoto, Japan) measuring light absorbance between 200 and 600 nm using 1 cm-path quartz cuvettes; and 2) fluorescence spectroscopy using the Shimadzu RF-5301 PC spectrofluorophotometer. Absorbance at 254 nm was used to monitor dissolved COD (Miroslav et al., 1983). Fluorescence Excitation-Emission-Matrix (EEM) spectra were collected with subsequent scanning emission spectra from 280 to 600 by varying the excitation wavelength from 250 to 450 nm at 5 nm increments. The software Panorama Fluorescence 2.1 was employed for handling EEM and Scilab (Digiteo Corp., France) was used to plot the matrix. Synchronous fluorescence spectra were also collected with an off set value equal to 20 nm. The tryptophan-like fluorescence at an excitation wavelength of 282 nm and an emission wavelength of 332 nm made it feasible to monitor the fate of soluble organic nitrogen (Sarraguça et al., 2009).

3. Results and Discussion

3.1. Comparison between MBR and CAS performances

The two laboratory-scales pilots (MBR and CAS) were run in parallel, fed with the described hospital effluent (table 1) during 65 days. Results reported in table (3) showed that the MBR was able to achieve very good organic removal efficiencies during the entire working period. Their removal efficiencies based on TSS, VSS, total and soluble COD ranged to 96.1 %, 87.9 % 86.9 % and 82.1 % respectively, whereas those with CAS were 89.1 %, 85 %, 77.4 % and 73.4 % respectively. As expected, the removal of solid compounds was more efficient with membrane bioreactor (MBR) than with conventional activated sludge treatment because the membrane act as a physical barrier.

Table 3
Organic pollutants removal efficiencies for CAS and MBR.

Efficiency of removal %	TSS	VSS	T COD	S COD	TN	SN
MBR	96,1	87,9	86,9	82,1	88,9	83,6
CAS	89,1	85	77,4	73,4	84,2	86,2

3.2. Occurrence and removal of PPCPs

The total efficiency of removal from water for each pharmaceutical compound was determined for both MBR and CAS according to Eq. (1):

$$\text{Removal \%} = 100 \times [(C1 - C2) / C1] \qquad \text{(Eq. 1)}$$

Where:

C1: concentration of a pharmaceutical compound in influent.

C2: concentration of a pharmaceutical compound in effluent.

Table 4 and Fig. 2A and B show the concentration of the studied phamaceuticals and the removal (%) for both reactors MBR and CAS, respectively and compared to data founded in scientific reviews, and represented by a vertical line between maximal and minimal removal data founded. A large disparity between the literature results was noticed, depending definitely of experimental set-up system or analysed processes. However, our results are in coherence with these values. It can be observed highest removal efficiency (95± 5%) or a complete removal for ketoprofen, paracetamol, ibuprofen caffeine, bezafibrate, fenofibrate, pravastatin, ramipril, atenolol, isosfamide and lohexol in the MBR and paracetamol, caffeine, fenofibrate, pravastatin, ramipril, isosfamide, estrone (E1) and estriol in the CAS. Roxithromycin has the most complex chemical structure of the target compounds and acts as an antibacterial agent. It has a moderate hydrophobic nature (log Kow=2.75) and a basic character. It was sparsely eliminated from both types of processes. The treatment of naproxen led to lower elimination rates of 48.28% in MBR and neither removal in CAS. It can be partially explained by a more stable chemical structure of this molecule. Sulfamethoxazole, possessing antibacterial properties, was eliminated by only 50% in the MBR and neither in CAS where its concentration increased during the 50 days of treatment, certainly due to the occurrence of conjugates in the effluent, which are partially hydrolysed.

A (MBR)

B (CAS)
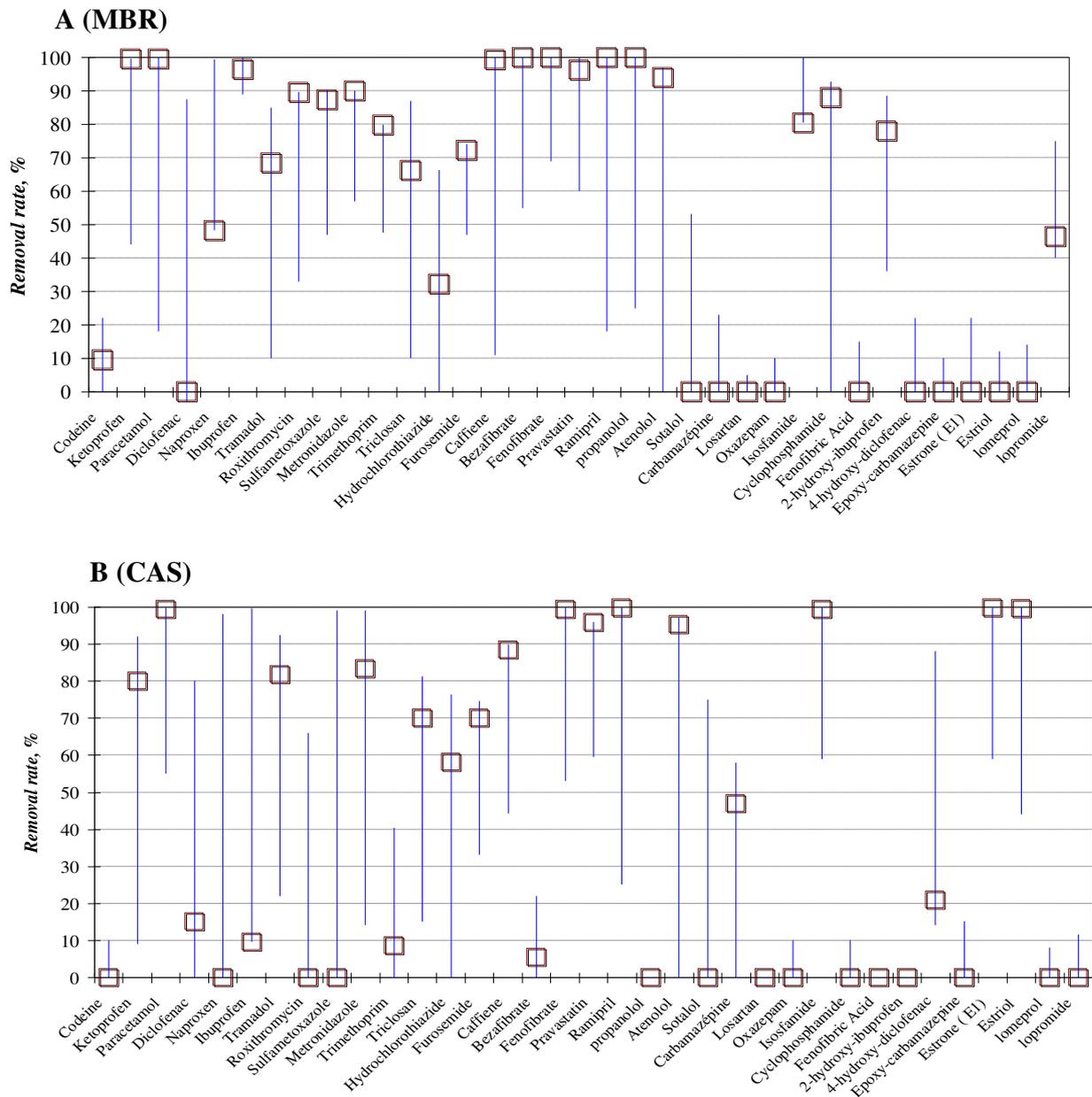
Fig. 2. Removal efficiencies (%) for 35 PPCPs in MBR (A) and CAS (B) reactors. Minimum and maximum removal efficiencies according to (Sipma et al., 2009).

The elimination of pharmaceutical compounds can occur through various mechanisms in MBR and CAS. Sorption onto sludge is one of the mechanisms involved as biodegradation or, for a short part, volatilisation. According to (Carballa et al., 2005), adsorption **r**efers to the hydrophobic interactions of the aliphatic and aromatic groups of a compound with fats present in the sludge or with the lipophilic cell membrane of the microorganisms (depending on their Kow value), while absorption refers to the electrostatic interactions of positively charged groups of dissolved chemicals with the negatively charged surfaces of the microorganisms (characterized by the dissociation constant pKa).

Table 4
Physico-chemical characteristics and average removal efficiencies of selected pharmaceuticals in CAS and MBR.

Pharmaceutical	MBR			CAS			Degradation Constant K *bio* (L/kg ss d)	Sorption constant Kd L/kg ss
	*Min. Rem. %	*Max. Rem. %	Rem. %	*Min. Rem.%	*Max. Rem. %	Rem. %		
Ramipril	18	99,5	100	25	99,9	99,8	n.d	n.d
propanolol	25	66,9	100	0	0	0	n.d	366
Bezafibrate	55	88	100	0	22	5,56	2,5	n.d
Fenofibrate	69	99,9	100	53	99,8	99,5	n.d	n.d
Paracétamol	18	100	99,60	55	99,9	99,4	80	n.d
Cafienne	11	99,9	99,28	44,2	89,9	88,37	n.d	n.d
Ibuprofen	89	99,9	96,39	52	99,7	9,64	20	7
Pravastatin	60	99,9	96,30	59,4	61,8	95,93	n.d	0
Atenolol	0	97	93,93	0	97	95,36	n.d	64
Metronidazole	57	90	90	14	99	83,33	n.d	n.d
Roxithromycin	33	75	89,6	0	66	0	0,2	300
Cyclophosphamide	0	92,6	88	0	10	0	n.d	n.d
Sulfametoxazole	47	90	87,4	0	99	0	6,2	300
Estriol	0	12	83,12	44	99,9	99,7	280	n.d
Isosfamide	89	99,9	80,5	59	99,9	99,4	n.d	n.d
Trimethoprim	47,5	66,7	79,8	0	40,4	8,57	0,15	200
Estrone (E1)	0	22	79,6	59	99,9	99,8	145	n.d
2-hydroxy-ibuprofen	36	88,5	77,95	0	0	0	n.d	n.d
Furosemide	47	74	72,22	33	74,5	70	n.d	n.d
Tramadol	10	85	68,52	22	92,4	81,8	0,11	111
Triclosan	10	86,9	66,20	15	81,2	70	n.d	n.d
Naproxen	71	99,3	48,28	0	98	0	1,5	13
lopromide	40	75	46,36	0	11,5	0	2,1	11
Fenofibric acid	0	15	22,10	0	0	8,15	0,5	5
Codeine	0	22	9,52	0	10	0	n.d	n.d
Sotalol	43,4	53,1	0	21,4	75	0	n.d	n.d
Carbamazepine	0	23	0	0	58	46,92	0,03	0,1
Losartan	0	5	0	0	0	0	n.d	n.d
Oxazepam	0	10	0	0	10	0	n.d	n.d
4-hydroxy-diclofenac	0	22	0	14	88	20,91	n.d	n.d
Epoxy-carbamazepine	0	10	0	0	15	0	n.d	n.d
lomeprol	0	14	0	0	8	0	n.d	n.d

*Refs: Sipma et al., 2009 ; Yu et al., 2008; Radjenovic et al.,2007 ; Joss et al., 2005 : Lee et al., 2003 : Gobel et al., 2007 ; Vieno et al., 2007; Radjenovic et al., 2009 ; comez et al., 2007 ; Heberer et al., 2002 ; Nakada et al., 2006 ; Santos et al.,2007 ; suarez et al ., 2005 ; Paxéus et al.,2004 ; Clara et al.,2004 ; Lishman et al.,2006.

(Göbel et al., 2007) studied the elimination of pharmaceuticals by MBR and CAS and concluded that the contribution of activated sludge adsorption in the case of pharmaceutical compounds was less than 6%, i.e., negligible, because this is within the analytical variance of the method.

In our study, sulfamethoxazole, which has a hydrophilic nature with two ionisable amine groups, can be present in an aqueous solution, in positive, neutral, or negative forms. At pH values between the pKa values of the compound (1.4 and 5.8), it predominates as a neutral species, while above the second pKa value of the compound (pH 5.8) it becomes a negatively charged specie (Göbel et al., 2007). These physicochemical properties give an indication that in the studied MBR system (pH 7.2) the sorption mechanism on sludge will play a negligible role, due to electrostatic repulsion between the negatively charged groups of the compound and the negatively charged surfaces of the sludge. Therefore, biodegradation can be considered as the main mechanism responsible for the removal. (Göbel et al., 2007) and that correspond with this study. Fig.3A shows the correlation between the walues of Kbio with the removal efficiency. Increasing the Kbio for the sulfamethoxazole was as indicator on the complete biodegradation which was correlated with high removal efficiency in CAS and MBR.

Considering the antibiotic compounds studied the highest removal efficiencies were observed for metronidazole (90 ± 5%) in the MBR and 83.3 ± 5% in the CAS. This can be partially explained by its basic character and reduced antibacterial potency compared to sulfamethoxazole and roxithromycin.

In the MBR, the Kd of ketoprofen, naproxen, ibuprofen, sulfamethoxazole, metronidazole, triclosan, hydrochlorothiazide, furosemide, carbamazepine, losartan, oxazepam, isosfamide, cyclophosphamide, Fenofibric acid, 2-hydroxy-ibuprofen, epoxy-carbamazepine, estrone (E1), estriol, iomeprol and lopromide showed a significant positive correlation with their removal efficiencies. (Fig. 3B and Table 4), suggesting that high removal efficiencies of these compounds in the wastewater treatments plant the important resulted in absorption on the activated sludge. The sorption phenomena seems to be a major removal mechanism in the MBR for ketoprofen, naproxen, ibuprofen, sulfamethoxazole, metronidazole, triclosan, hydrochlorothiazide, furosemide, carbamazepine, losartan, oxazepam, isosfamide, cyclophosphamide, fenofibric acid, 2-hydroxy-ibuprofen, epoxy-carbamazepine, estrone (E1), estriol, lomeprol and lopromide (Golet et al., 2003; Lindberg et al., 2006).

Several assumptions may be made to explain the difference between CAS and MBR: (1) a higher SRT in the MBR allows a better degradation of non-easily biodegradable molecules (as pharmaceuticals) and (2) could also enhance the development of slowly growing populations, (3) a higher concentration of no-flocculating and dispersed organisms in MBR probably aids the degradation of molecules in the supernatant due to reduction of mass transfer limitation,

(4) one of the reaction of bacteria to an environmental stress is the production of EPS which can increase the sorption characteristics between sorbable molecules and sludge.
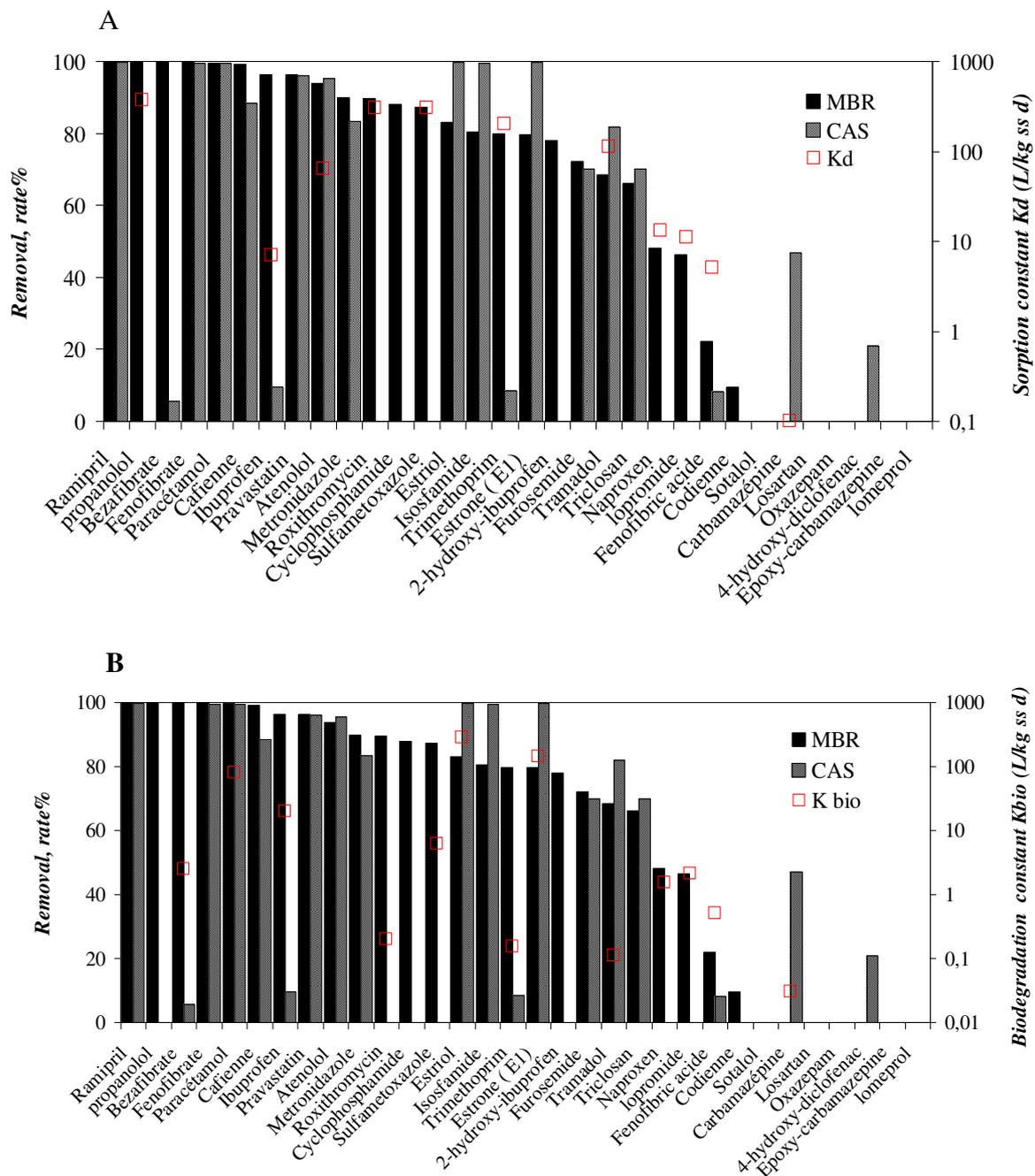
Fig. 3. Removal efficiency with the Kbio (A) and the Kd (B).

3.2 EPS measurement

Samples of flocs were qualitatively observed using confocal laser scanning microscopy (CLSM) to characterize the extracellular polymeric substances (EPS). Visualization of a flocs collected in the CAS and BRM reactors after a 2, 18, 30, and 48 days of exposure time to hospital effluent is presented in Figure 4 after statistical treatment. The relative quantity of

total EPS increased in the CAS during the 30 first days of experiment, and then decreased at the end; this may show some acclimation of the biomass to the specificity of the effluent. On the other hand, in the MBR, the relative quantity of EPS increased continuously during the time of experiment, leading to an accumulation on the media, and, accordingly, to a decrease of the membrane permeability. The involving of cellular lysis in the occurrence of EPS was also visualized by confocal microscopy stained with fluorescent viability indicator (Fig. 4). The relationship between the evolutions of alive cells and EPS seems more evident in the CAS, where the percentage of EPS is closed to the percentage of alive cells (except at 30 days). It is not the case in the MBR, confirming that EPS could be a by-product due to the type of process.
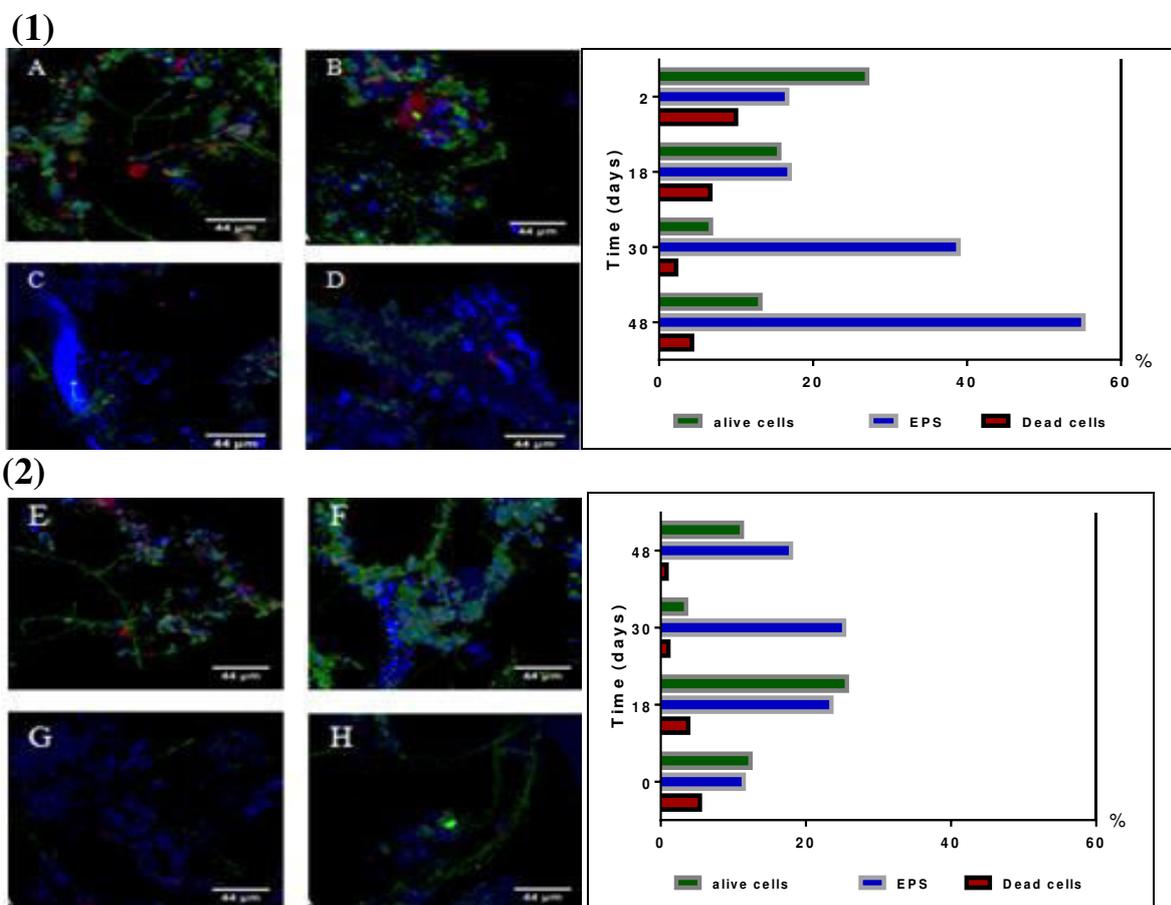
Fig. 4. Evolution of alive and dead cells and EPS in MBR (1) and CAS (2)

To confirm these results, three-dimensional EEM spectroscopy was applied to characterize the soluble EPS from both MBR and CAS sludge supernatant. Three peaks were readily identified from EEM fluorescence spectra of effluents from BRM-EPS and CAS-EPS during 60 days of treatment (Fig. 5. A, B). The first main peak was identified at excitation/emission wavelengths (Ex/Em) of 240/300–310nm (Peak I), while the second main peak was identified

at Ex/Em of 250–275/340–350nm (Peak II). These two peaks have been described as protein-like peaks, in which the fluorescence is associated with the aromatic amino acid tryptophan (Baker, 2001; Chen et al., 2003; Yamashita and Tanoue, 2003; Baker and Inverarity, 2004). Compared with the fluorescence peak location of proteins reported previously (276–281/340–370nm) (Baker, 2001), the locations of Peak II for the two EPS showed a blue shift. A third peak was located around Ex/Em = 280-300/380–400nm (Peak III). A similar fluorescence signal has also been observed for natural dissolved organic matter and is described as visible humic acid-like fluorescence (Coble, 1996).
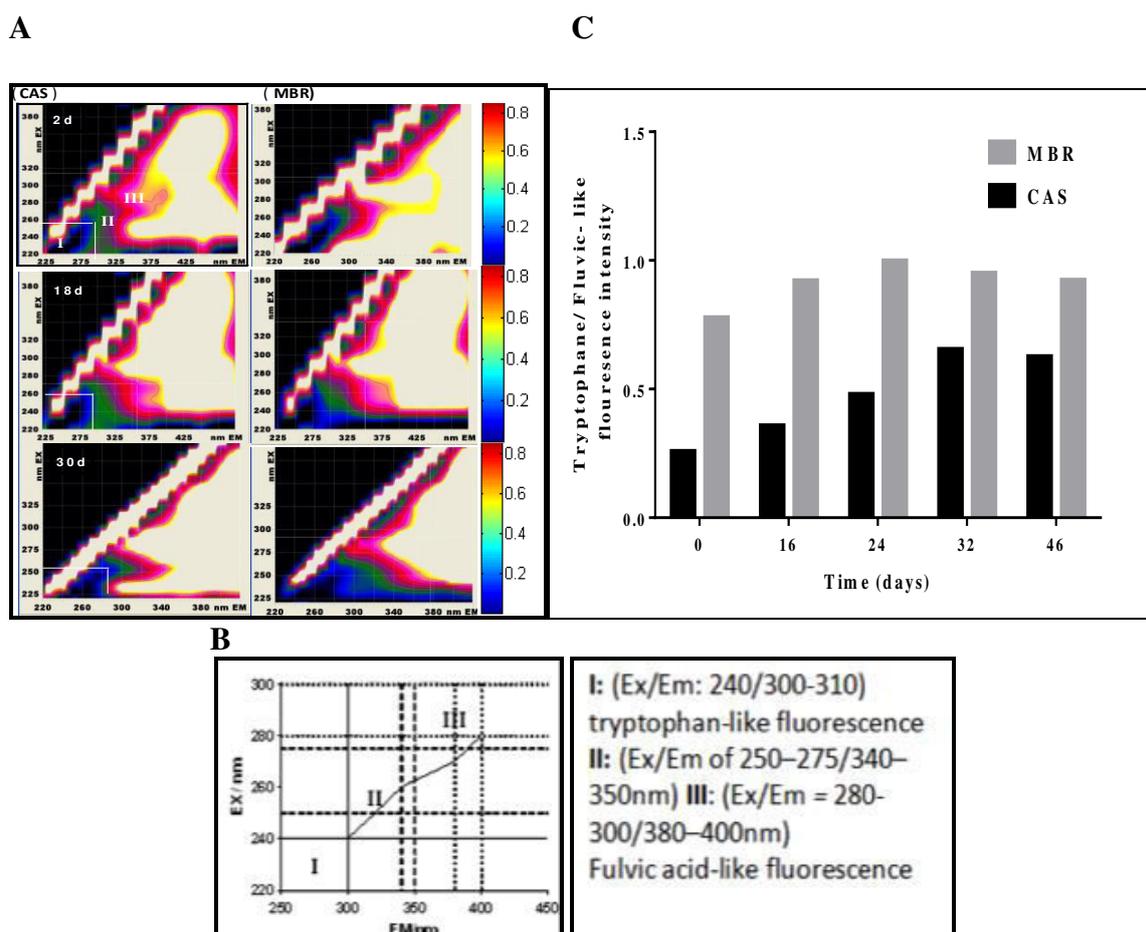
Fig. 5. (A, B) EEM fluorescence spectra of the soluble-EPS. (C) The ratio tryptophan/fulvic-like fluorescence intensity versus time in CAS and MBR reactors treating the hospital wastewater.

The change in the relative fluorescence intensity of the peaks during activated sludge treatment gave interesting information about the composition of sludge and the change in the EPS structure. The ratio tryptophan-like/fulvic-like fluorescence intensity was considered as an indicator of the biologic state of wastewater. During the first 40 days the ratio between the tryptophan-like fluorescence and the fulvic- like substances fluorescence was increased from

0.25 to 0.65 for the CAS and from 0.79 to 1 for the MBR (Fig. 5C) indicating an increase in proteins concentration in both case.

Synchronous fluorescence with an offset value of 20 nm permitted to measure the change in the peak I, peak II and peak III intensity for both CAS and MBR reactors (Fig. 6A and B). Important according between concentrations of the EPS compounds measured in both fluoremetric and chemical analyses in both MBR (A) and CAS (B) reactors and that confirms the impact of the hospital effluents in increasing concentration the EPS during the 40 days of experiment.

A

B
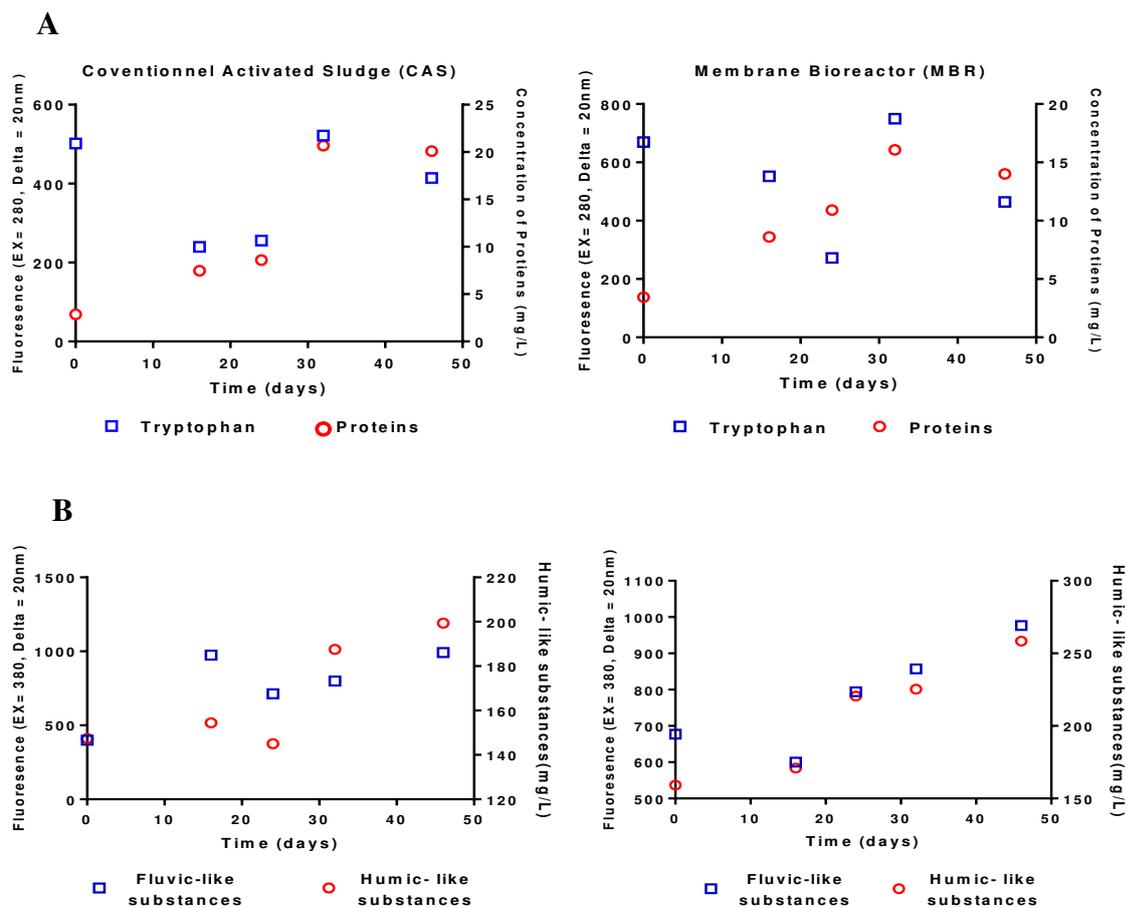
Fig. 6. C The relation between the chemical dosage for the proteins and humic-like substances and tryptophan-like fluorescence (λ exc= 280 nm, Δ=20 nm), fulvic-like fluorescence (λ exc = 365 nm, Δ= 20 nm) versus time during the time of the (A) MBR and (B) CAS

To quantify the EPS, the total EPS composition (PN, PS, HA) were analysed by biochemical analyses and their evolutions were represented in the Fig. 7. Significant increasing of total EPS was found during the experiment in both MBR and CAS reactors. Protein concentration was very low and its increasing in supernatant concentrations in MBR and CAS was significant since the days 35 (from 4mg/L to 20 mg/L).

In a general way, concentration of proteins, polysaccharides and humic-like substances were equal or higher in the MBR than in the CAS, especially after 30 days of operating (20 mg/L, 70 mg/L, 300 mg/L respectively in the MBR against 20mg/L, 40mg/L and 200mg/L in the CAS).
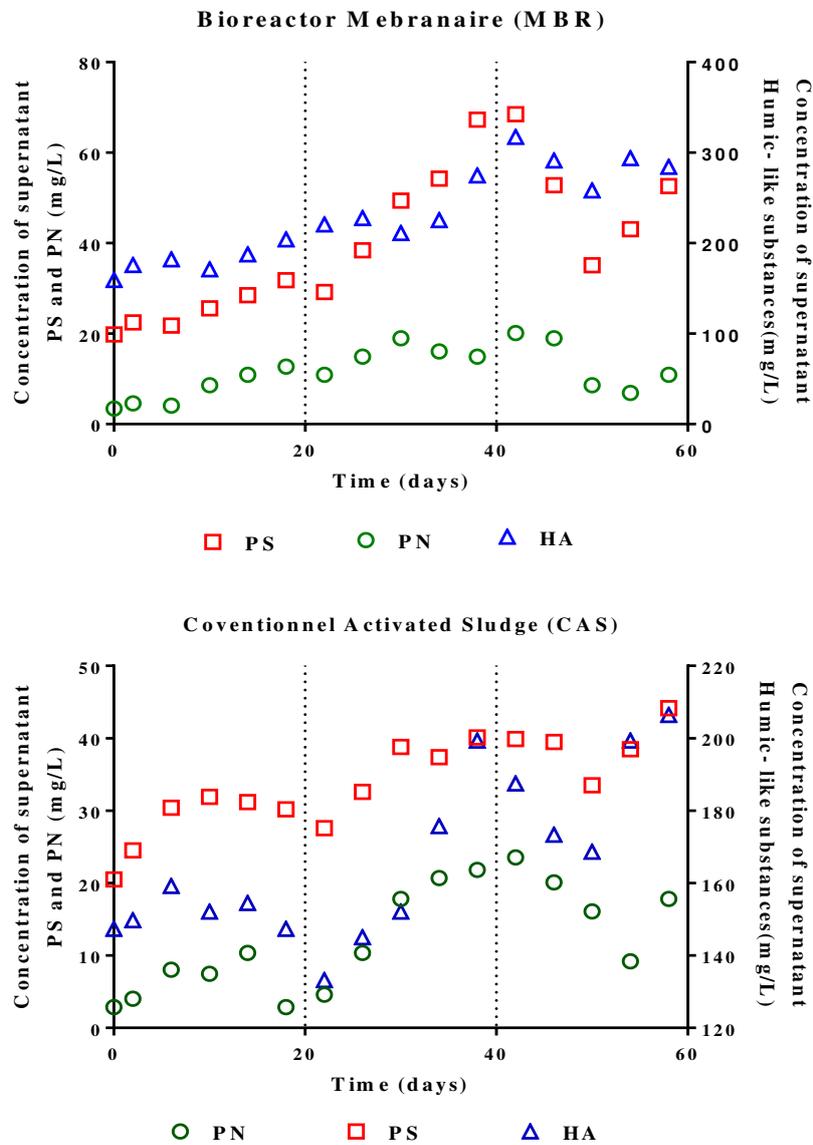
Fig. 7. EPS concentration variation in supernatant (MBR and CAS).

These compounds could be directly brought in by the influent and/or produced in the reactor (Guo-Ping Sheng et al., 2010). In the first case, their concentration in the supernatant depends on their adsorption onto microbial flocs, their removal by sludge withdrawal and their passage through the membrane in MBR (Delgado et al., 2010). In the second case, EPS is constitutive of the bacterial floc and the product of an environmental stress. A simple mass

balance for each compounds showed that if humic-likes substances concentration resulted of the quality of the influent, proteins and polysaccharides concentrations were the result of microbial metabolism.

Moreover, it has been shown that the presence of pharmaceuticals compounds stimulates the survival mechanisms of microorganisms and the production of EPS with a slightly higher production of polysaccharides than proteins (A.C. Acella et al., 2009). It can thus be supposed that the higher concentration of EPS in MBR compared to CAS was also linked to cake layer retention of the membrane.

4. Conclusion

The MBR was able to achieve good organic removal efficiencies by comparison with the CAS. Despite the low concentration studied, the pharmaceutical compounds modifie the characteristics of the biological matrix. Their occurrence stimulated the mechanisms of survival (higher production of EPS. Fouling potential seems to be linked more closely to polysaccharides than other EPS. Simultaneously, confocal laser scanning observations and three-dimensional EEM spectroscopy showed significant modifications of sludge morphology. (Higher production of soluble EPS). The MBR presented higher removal efficiencies for pharmaceuticals by compared with the CAS.

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Amélioration des performances de systèmes à boue activée couplés à une membrane d'ultrafiltration interne ou externe par ajouts de supports bactériens.

Afin d'améliorer les performances des réacteurs biologiques couplés à un système de séparation membranaire, des supports bactériens synthétiques ont été ajoutés à la liqueur mixte afin de favoriser la croissance d'un biofilm. Deux configurations ont été testées (A et B) :

- un système à membrane immergée, fonctionnant selon les modalités des bioréacteurs à membrane (recyclage interne)
- un système à membrane externe, traitant une eau partiellement décantée, et fonctionnant comme traitement tertiaire. Les résultats de cette deuxième configuration ont été publiés dans Chemical Engineering Journal.

A- Performances d'un bioréacteur membranaire à biofilm traitant un effluent hospitalier par ajout d'un support bactérien (cette partie est écrite pour le publier en « Desalination journal »)

Des supports bactériens ont été ajoutés dans la liqueur mixte d'un réacteur à membrane immergée (BAM) traitant un effluent hospitalier, afin de le transformer en bioréacteur membranaire à biofilm (MBBR) et améliorer ces performances en terme d'élimination de composés pharmaceutiques. Ainsi, après 60 jours de fonctionnement en BAM, les supports ont été ajoutés et le système suivi pendant 60 jours également. D'une manière globale, les rendements d'élimination pour les paramètres classiques (DCO, MES, MVS, et NT) ont été améliorés lors du passage en MBBR. Dans le cas des composés pharmaceutiques, si d'une manière générale les taux d'abattement sont meilleurs en MBBR qu'en BAM (Tramadol, sulfaméthoxazole, triméthoprime, naproxène, triclosan, métoprolol, sotalol, losartan, carboxyle ibuprofène, ibuprofène 2-hydroxy, époxy carbamazépine, 4 androstene-3, 17-dione et ioméprol) des exceptions ont été mesurés (propanolol 100% à 25%).

Comme lors des travaux précédents, les EPS ont été analysées par la méthode biochimique et par microscopie confocale, couplée avec une estimation de la viabilité cellulaire. Après une augmentation de la concentration de protéines, de polysaccharides ou d'acides humiques-like lors du fonctionnement en BAM, leurs concentrations dans la phase liquide ont brutalement diminuées pour se stabiliser. Seule la concentration en acides humiques-like augmente de nouveau, certainement liée à l'alimentation.

Une des répercussions de l'ajout de garnissage a été de diminuer le colmatage membranaire et donc de réduire le nombre de lavage en stabilisant la perméabilité membranaire.

B- Amélioration des performances d'un système à boue activée couplés à une membrane d'ultrafiltration par ajouts de supports bactériens.

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Un réacteur à boue activée couplé à une membrane d'ultrafiltration (BAM) a été mis en place pour le traitement d'un effluent hospitalier et suivi pendant 75 jours en terme de performance d'élimination de composés pharmaceutiques et, comme précédemment, de modification structurelle des flocs.

Comme précédemment des supports bactériens ont été ajoutés dans la liqueur mixte du bassin aéré afin de transformer le réacteur en bioréacteur membranaire à biofilm (MBBR) et suivi pendant 2 mois supplémentaires sur les mêmes performances.

D'une manière globale, les rendements d'élimination pour les paramètres classiques (DCO, MES, MVS, et NT) ont été améliorés lors du passage en MBBR.

Dans le cas des composés pharmaceutiques, si d'une manière générale les taux d'abattement sont meilleurs en MBBR qu'en BAM (Tramadol, sulfaméthoxazole, triméthoprime, naproxène, triclosan, métoprolol, sotalol, losartan, carboxyle ibuprofène, ibuprofène 2-hydroxy, époxy carbamazépine, 4 androstene-3, 17-dione et ioméprol) des exceptions ont été constatées (propanolol 100% à 25%).

Les différentes hypothèses avancées, validée par des études antérieures, sont :

- l'augmentation du temps de séjour des boues lié à la présence d'un biofilm néoformé sur les supports ajoutés
- l'augmentation de la biomasse dans les structures de biofilm
- l'augmentation des phénomènes de sorption sur les biofilms supportés. .

Comme lors des travaux précédents, les EPS ont été analysées par la méthode biochimique et par microscopie confocale, couplée avec une estimation de la viabilité cellulaire.

Après une augmentation de la concentration de protéines, de polysaccharides ou d'acides humiques-like lors du fonctionnement en BAM, leurs concentrations dans la phase liquide ont brutalement

diminuées pour se stabiliser. Seule la concentration en acides humiques-like augmente de nouveau, certainement liée à l'apport de ces composés par l'alimentation.

Le suivi des évolutions de la pression transmembranaire et du flux de perméat a montré que l'ajout de garnissage a largement stabilisé les évolutions de ces deux paramètres. Une des conséquences de l'ajout de garnissage a donc été de diminuer le colmatage membranaire et ainsi de réduire le nombre de lavage en stabilisant la perméabilité membranaire. Cette diminution est mise en relation avec les productions des différents EPS.

A- Application of membrane biofilm bioreactor (MBBR) for hospital wastewater treatment: Performances and Efficiency for Organic Micropollutant Elimination

1. Introduction

The use of membrane bioreactors (MBR) is emerging as an attractive technology for hospital wastewater treatment with considerable advantages over conventional treatment methods (Arnot et al. 1996). The bioreactor which combines membrane system and biological treatment processes into a single unit is designed to remove particulate, colloidal and some dissolved substances from the solutions (Chang et al. 1998). The membrane separation technique could be used to avoid a problem of non-settling sludge, to replace a secondary clarifier, and to obtain a high effluent quality and a compactness of treatment plants (Visvanathan et al., 2000).

Nevertheless, membrane fouling is one of the main drawbacks of this technique and it is generally accepted that fouling reduces the performance of membrane. To overcome membrane fouling due to the cake resistance, a number of techniques have been explored: backwashing, jet aeration, operation below critical flux, addition of coagulants (Lee et al. 2000). Most of the studies have focused on minimizing the cake formation on the membrane surface, but another way is to use a support media in the bioreactor to fix the biomass and there by to limit the primary sources of cake layer. When the fouling occurs, a thick gel layer and cake layer are formed on and into the membrane, causing the decrease of the permeate flux and the increase of the operating costs due to needs for cleaning or replacing the membrane.

Fouling is usually attributed to a number of parameters, such as sludge particle deposition, adhesion of macromolecules such as extracellular polymeric substances (EPS) and pore clogging by small molecules (Bouhabila 1996). Soluble EPS (soluble macromolecule and colloid) can enter the membrane pores and then build up on the pore wall, leading to a reduction of total section area of membrane pores causing pore plugging into membrane and increasing the membrane resistance (Lukas et al. 2002). The membrane performance can be monitored through a number of factors such as membrane fouling, EPS production,

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treated effluent quality, biomass characteristic and microbial activity (Lee et al. 2003; Kim et al. 2001).

A number of studies have been experimentally conducted on membrane fouling (Chang et al. 1998; Nagaoka et al. 1996; Ognier et al. 2002) investigating an attached growth bioreactor with fixed support media to minimize the fouling in submerged MBR.

(Basu et al. 2005) studied the effect of support media in integrated bio filter submerged membrane system, and membrane fouling rate and water quality parameters were of interest. It was found that the membrane fouling rate doubled in the absence of support media. The authors also suggested that the support media enhanced the membrane surface scouring and the bio film growth on the support media, which improved the removal efficiency. The comparison, reported in this paper, was intended to check whether the attached growth treatment of effluent hospitals could increase performance the MBR in removal the organic pollutants and the micropollutants. MBR was used to treat the hospital effluent and to evaluate its performance for the MBR with supports media or without supports media but not in term of performances but also the changes in EPS concentration.

2. Materials and Methods

2.1. Study area

The hospital effluent (HE) samples used in this study were collected from the sewerage system (black water) which comprises only sewers from clinical activities of the hospital. Average characteristics of wastewater and activated sludge used as inoculums during the experiments are detailed in the (table 1).

Table1 Show physicochemical characteristics of the hospital effluents (HE) and activated sludge (AS).

	COD (mg/L)		N (mg/L)		TSS (g/L)	VSS (g/L)
	Total	Soluble	Total	Soluble		
HE	412.5± 5	173.5 ±5	128.9±5	95± 5	0.199	0.091
AS	1201± 5	285± 5	-	-	6.214	1.35

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2.2. Membrane bioreactors (MBR)

Submerged membrane bioreactor (MBR) having 27 L of working volume were used under a laboratory scale. The reactor had a rectangular cross section and was separated into two compartments by a vertical holed baffle plate to prevent the moving media from contacting the membrane module and protecting it from breakage. The MBR system consisted of bioreactor. Hollow fiber membrane module was submerged in bioreactor shown in (Fig1). The characteristics of the membrane used in this work are listed in (Table 2).
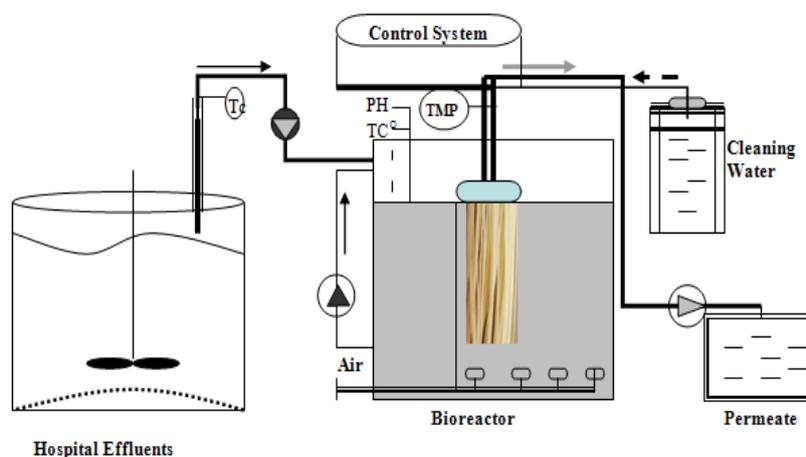
Figure 1 Schematic diagram of membrane bioreactor

Table 2 the characteristics of the membrane used in this work

Item	Membrane characteristics
Model	STNM424
Membrane material	Polyethylene (coating with hydrophilic)
Membrane configuration	Hollow fiber
Pore size	0.05μm
Surface area	1 m2
Manufacturer	Rayon Co., Ltd (Japan)

Aeration was done through diffusers at the bottom of the reactor to provide oxygen for biomass growth as well as shear to reduce cake formation at membrane surface.

Dissolved oxygen levels were maintained between 2 and 4.5mg O2/h. The membrane permeate was continuously removed by a peristaltic pump under a constant flux (1.8 L/h) constantly monitoring the trans-membrane pressure (TMP) build-up which indicates the extent of membrane fouling and under intermittent operation   mode in a automatic cycle for 10 minute of production (on), and 45 seconds for physical water cleaning operation (off) by using a integrated timer. The membrane cleaning process was temporarily required when the membrane was clogged, which was indicated by an increase in the transmembrane pressure (TMP) up to ~26 kPa. The TMP value was measured using a U-shaped Hg manometer. The hydraulic retention time (HRT) ranged from 15 to 20 h. Temperature was from 14 to 16 C° and pH was from 7 to 8. The sludge retention time (SRT) was around 15 days. The bioreactor was run for 95 days in two operations the first begin from 1 to 60 days without the biofilm supports media (MBR) and the second from 60 to 120 days with the biofilms supports media (MBBR). See table 3 to know more about the characteristics of supports.

Table 3 Characteristics of supports media

Foumisseur	Stohr
Activated area m²/m³	660
Diameter (mm)	12,2
Length (mm)	12
Density	0,95-098
Materiel	PEHD
Weight (Kg/m²)	150

2.3. Analytical methods

Wastewaters and sludge physicochemical characteristic measurements were done every two day. Measurements of total and volatile suspended solids (TSS and VSS) were done according to the normalized method (AFNOR, NF T 90-105). Chemical Oxygen Demand (COD) was measured by the closed reflux colorimetric method (ISO 15705:2002), and total nitrogen (TN) was assessed using the alkaline per sulfate digestion with colorimetric reactive (Hatch company). The COD and TN were carried out on both total and soluble fraction (after

samples filtrated at 1.2μm). Ionic species in solution were determined on samples filtrated at 0.22μm using ion chromatography *(Dionex 120)* according to the standard method *(AFNOR, NF EN ISO 10304-1).* The used detector was conducted metric, and the analytical error was ±5%.

2.4. Extracellular polymeric substances (EPS)

The analysis of EPS in biomass was made through a thermal extraction method. The mixed liquor of activated sludge was centrifuged at 4000 rpm for 20 min and T= 4 C° in order to remove the soluble EPS from bound EPS. After collecting the soluble EPS, the remaining pellet was washed and re-suspended in saline water (0.9% NaCl solution). The extracted solution was then separated from the sludge solids by centrifuging under similar conditions (4,000 rpm for 20 min and T= 4 C°), the supernatant obtained at this stage being referred to as bound EPS solution.

2.4.1. Analysis of total protein, humic substances -likes and polysaccharides

Protein content, expressed in mg equivalent of bovine serum albumin per gram of VSS (mg/ L for the soluble polymer), was determined according to the method of *Lowry et al. (1951)* with a correction for the humic substances .Humic substances- likes were measured with the Folin-Ciocalteau phenol reagent in the same trial as the protein by omitting the CuSO4. Results were expressed in mg equivalent of humic substances- likes per gram of VSS (mg/L) for the soluble polymer. Polysaccharides were determined according to the method of *Dubois et al. (1956)* and the results expressed in mg equivalent of glucose per gram of VSS (mg/ L) for the soluble polymer.

2.5. Confocal laser scanning microscopy

To characterize the extracellular polymeric substances of sludge, samples of flocs were observed using 3D-CLSM combined with a fluorescent viability indicator (Backlight®Bacterial Viability Kit, Molecular Probes) allowing visualization of isolated stained cells in the three-dimensional structure of flocs (damaged or not). For the image series a Zeiss LCM 710 NLO confocal microscope equipped with laser diode was used with an HCX 5×0.5. The band width of the detected fluorescence wavelengths has been optimized to uniquely channel the

maximum emission in sequential mode to avoid potential cross-talking. Fluorescence emissions were recorded within 1 airy disk confocal pinhole opening and 1024 × 1024 images at a 1.36-m (x, y) pixel size were obtained. Instead of selecting a constant step size in the vertical direction, the step size was determined by choosing start and end points in the z-direction of the flocs, and by then selecting a number of optical sections.

2.6. Dosage the Pharmaceuticals and Personal Care Products (PPCPs) in the wastewater

Two different analytical methods were applied to determine the concentration levels of the PPCPs in the wastewaters samples. Water samples were enriched by liquid-solid phase (SPE) by using Oasis HLB cartridges (6ml, 200mg) from waters. The SPE extracts were injected in liquid chromatography mass spectrometry (LC-MS/MS). Acquisition was performed in selected reaction monitoring (SRM) mode and tow transitions (quantification, confirmation) were obtained for each compound. Quality control (QC) was assured by measuring two transitions for each analyze and each internal standard, comparing retention time of analyze with the retention time of the internal standard in each sample, duplicates, numerous blanks, and QC standards. In global analytical error was about ~ ± 10µg/L. (this analysis was occurred in IANSCO laboratory, Poitiers, France).

3. Results and Discussion

3.1. Reactor operation and performance

The treatment of a hospital effluent has been running during 120 days, with an operating cycle without biofilm supports during the first of 60 days (MBR) and the addition of these supports the 61 th day (MBBR).   Lower values in term of total and soluble COD, TSS,, VSS and total N removal were observed in the (MBR) compare to the MBBR (table 4). These results demonstrated that the presence of supports media allowed an increase of global microbial activity due to the increase of biomass concentration on the support and of the SRT of fixed organisms.

Table 4 showed that the removal efficiencies

Efficiency of removal %	TSS	VSS	Total Phase		Soluble Phase	
			COD	N	N	COD
MBR	97,3± 1%	87,8± 1%	80,4± 5%	78± 5%	85,3± 5%	71,6± 5%
MBBR	99± 1%	97,6± 1%	94,2± 5%	84,9± 5%	84,3± 5%	90,7± 5%

The global removal efficiency (including adsorption and membrane fouling) was determined according to Eq 1:

$$Removal\ \% = 100 \times [(C_1 - C_2) / C_1]$$

Where:

C1 the experimental concentration determined in each reactor influent by analysis.

C2 the experimental concentration determined in each reactor effluent by the analysis.

Results measured are illustrated in Figure 2. The overall permeability for MBR at 15 days of operations is not essentially the same by compared with the MBBR. Based on this analysis, the performance of the membrane filtration in MBBR is significantly affected by addition the biofilms supports media in the reactor. In our hypotheses the biofilm could be fixed on the supports and that decreasing concentration the soluble EPS free in the reactor.

This hypothesis was confirmed with the Figure 3 which illustrated increasing fouling rate with concentration the soluble EPS in MBR. Au contrary, the fouling rate was from 0.01 to 0.04 in MBBR although the increasing of concentration the soluble EPS. The results confirm the importance of biofilm supports media as a means to mitigate fouling in immersed membrane systems. These results are in agreement with findings from other studies; (Invanovic et al., 2011; Basu et al., 2006).
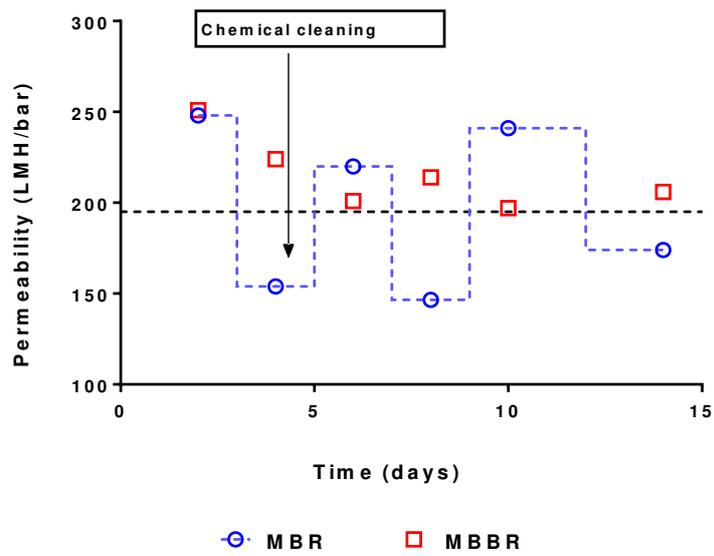
Figure 2 Example on changes of overall permeability during the experiment in MBR and MBBR.
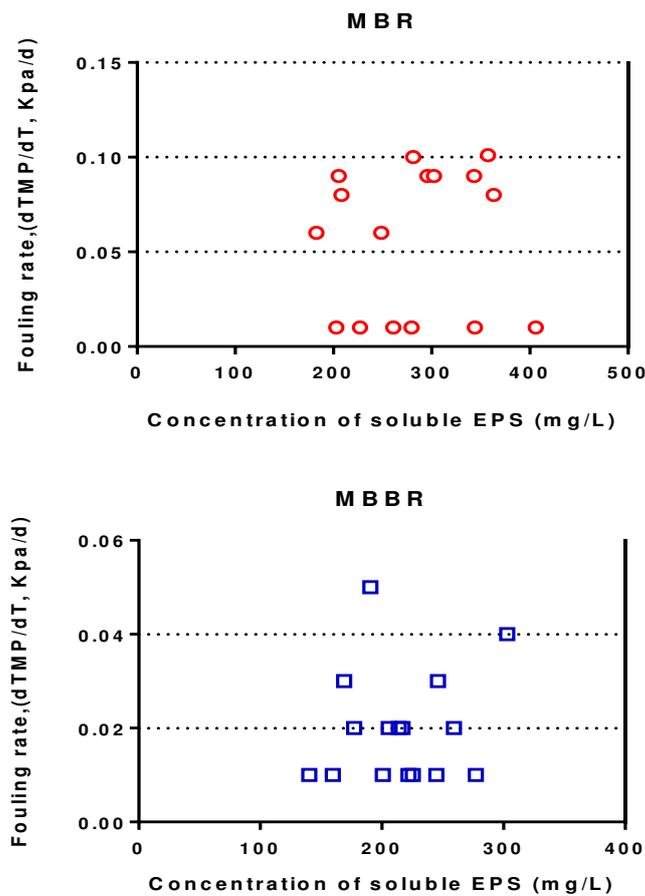
Figure 3 correlations between soluble EPS and calculated fouling rate in both MBR and MBBR reactors.

3.2. Occurrence and removal of Pharmaceuticals and Personal Care Products (PPCPs) in hospital wastewaters

The different mechanisms responsible for pharmaceutical compounds removal from hospital wastewater a biological system are sludge sorption on organic matter and biodegradation by microorganisms present in the wastewater (Cho et al. 2004). The total removal efficiency (sludge sorption+ biodegradation+ membrane retention) of 27 pharmaceutical compounds was determined for both MBBR and MBR and compared to bibliography (table5). The concentrations of the various pharmaceutical compounds and some transformation products during the study period were determined. (Table5) shows the concentration of the PPCPs in influent and effluent for both reactor MBBR and MBR. It can be clearly observed the highest removal efficiency (90±5%) or complete removal of ketoprofen, paracetamol, ibuprofen, caffeine, metronidazole, pravastatin, atenolol in both BRM but Tramadol, sulfamethoxazole, trimethoprim, naproxen, triclosan, metoprolol, sotalol, Losartan, carboxyl ibuprofen, 2-hydroxy ibuprofen, epoxy carbamazepine, 4 androstene -3, 17-dione, and iomeprol could be high removed by MBBR, while lower removal by MBR was found for them. The total removal efficiency (sludge sorption+ biodegradation + membrane retention) of each pharmaceutical compound was determined MBR according to

Eq 2:

$$Removal\ \% = (C2-C1) / C2 \times 100$$

Where:

C1: the experimental concentration determined for each pharmaceutical compound in each reactor influent by LC / (MS-MS) analysis.

C2: the experimental concentration of each pharmaceutical compound in each reactor effluent by the LC / (MS-MS) analysis

Table 5 the concentration of pharmaceutics compound in influent and effluent for both MBBR and MB

Pharmaceutical compound	Influent (µg/L)	Removal (±5%)		References
		MBR	MBBR	
Ketoprofen	15± 10	99	97	(30%) (Jelic et al. 2011)
paracetamol	310± 10	95	98	
Diclofenac	0,11± 10	42	68	(5–45 %) (Joss et al. 2005)
Naproxen	0,32± 10	34	95	(55-85 %) (Joss et al. 2005)
Ibuprofen	17± 10	99	95	(90-100 %) (Joss et al. 2005)
Tramadol	3± 10	22	92	
sulfamethoxazole	21± 10	33	91	<85 % (Jelic et al. 2011
Metronidazole	7,3± 10	86	95	15–18 % (Jelic et al. 2011
Trimethoprim	10± 10	10.5	89	90% (Göbel et al. 2007)
Triclosan	0,14± 10	22	100	
Hydrochlorothiazide	0,96± 10	92	45	(0-100 %) (Jelic et al. 2011
Furosemide	12± 10	83	52	35–40 % (Jelic et al. 2011
Caffeine	130± 10	96	98	
Pravastatin	0,74± 10	95	95	45 % (Jelic et al. 2011
metoprolol	0,09± 10	42	95	35 % (Jelic et al. 2011
Propanolol	0,24± 10	100	25	
Atenolol	1,5± 10	93	97	70 % (Jelic et al. 2011
Sotalol	2,3± 10	31.8	95	10% (Jelic et al. 2011
Carbamazepine	1± 10	58	83	< 40 % (Joss et al. 2005)
Losartan	0,2± 10	45	99	30 % (Jelic et al. 2011
Oxazepam	0,55± 10	86	15	60 % (Jelic et al. 2011
Fenofibric acid	5,6± 10	99	71	
Carboxyl ibuprofen	110± 10	51.9	96	80 % (Joss et al. 2005)
2-hydroxy ibuprofen	38± 10	29.6	98	
Epoxy carbamazepine	0,67± 10	69	97	40% (Joss et al. 2005)
4 androstene -3,17-dione	0,27± 10	56	99	
Iomeprol	960± 10	55	96	60-80 % (Jelic et al. 2011

3.3. Evolution of Extracellular polymeric substances (EPS) and their distribution in the sludge

The EPS production under our operating conditions was determined in terms of total and soluble EPS through the extraction method. (Fig 4) shows the concentrations of proteins, polysaccharides and the substances humic substances- likes in the soluble EPS. The concentrations of proteins and polysaccharides in the soluble phase increased during the 40 first days of operating in the MBR, from 4 mg/L to 20 mg/L and from 20 mg/L to 70 mg/L respectively, before a decrease certainly due to the chemical washing. The value of these concentrations could be due to the occurrence of pharmaceuticals compounds (Sheng et al., 2010) or, for a part, directly brought in by the influent. Therefore, their concentrations in the supernatant depend on their adsorption onto microbial flocs, their removal by sludge withdrawal and their passage through the membrane in MBR (Delgado et al; 2010). While these concentrations are globally constant for the MBBR. However the concentration of the soluble proteins and soluble polysaccharides was lower in the MBBR. (It was between 4 to 8 mg/L and 5-20 mg/L respectively). As it is known, the occurrence of biofilms increases the concentration of EPS fixed and this EPS are part of their structure.

On the other side, the concentrations of humic substances-like acids in the two systems are closed, with a slow increasing. But concentrations of soluble humic substances –like were decreased directly after addition the biofilms supports media, and then it was increased slowly throughout the experiment.
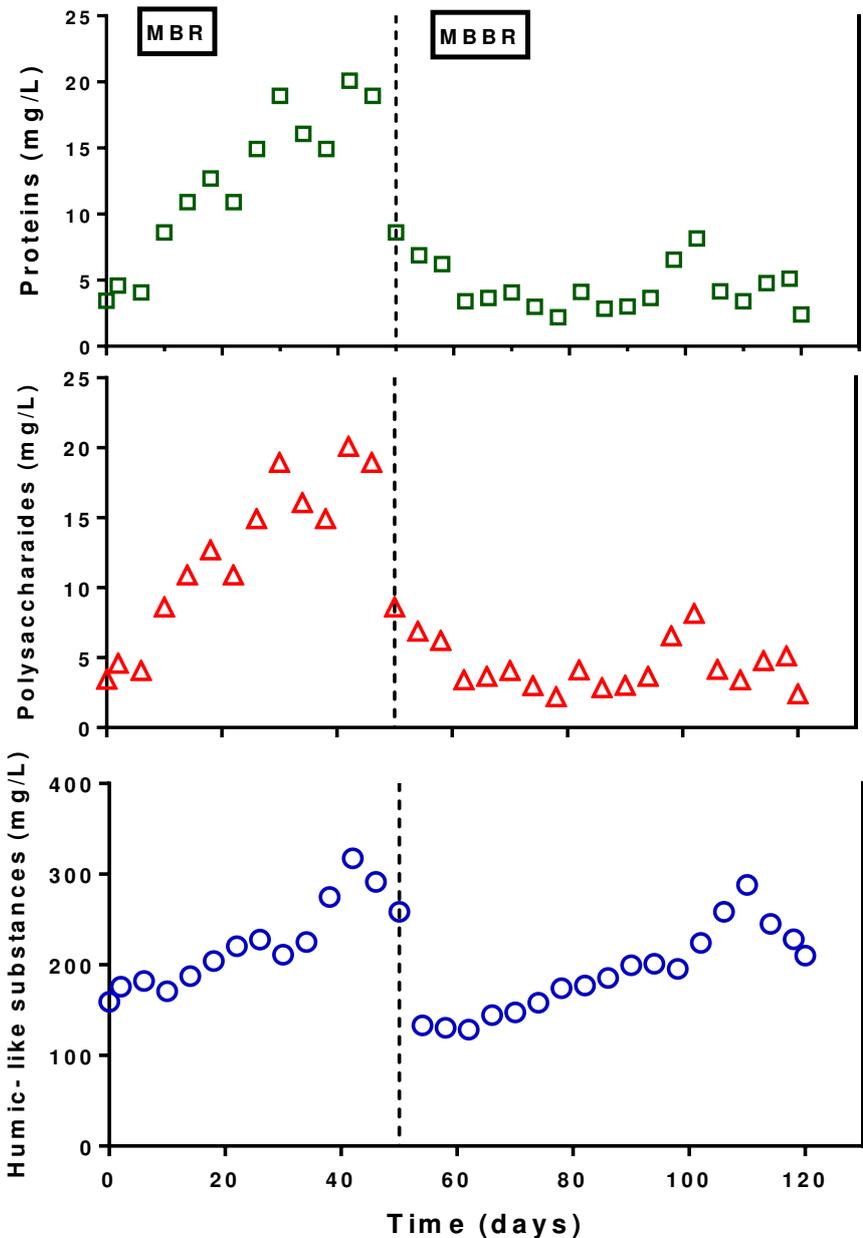
Figure 4: Variation of concentration of proteins, polysaccharides and humic substances-like during the experiment for tow reactors (MBBR and MBR)
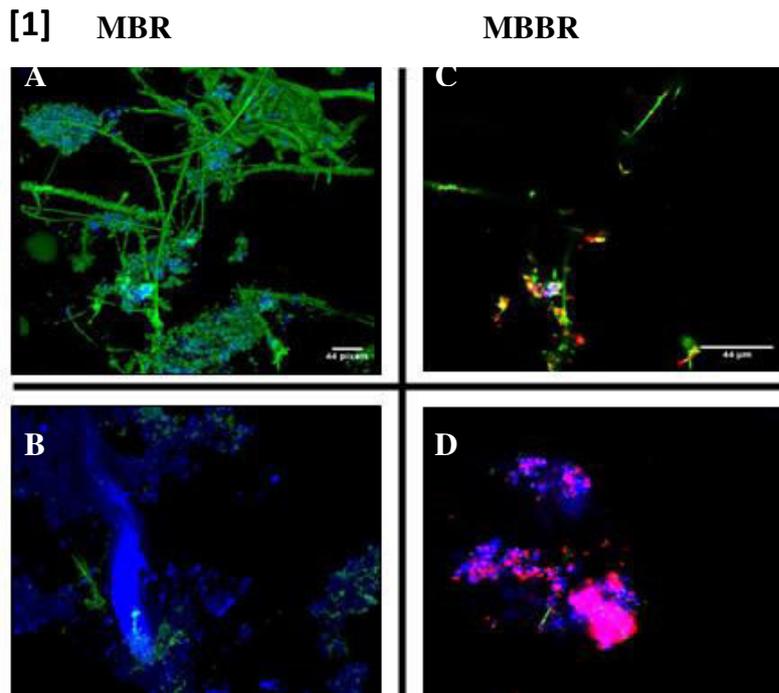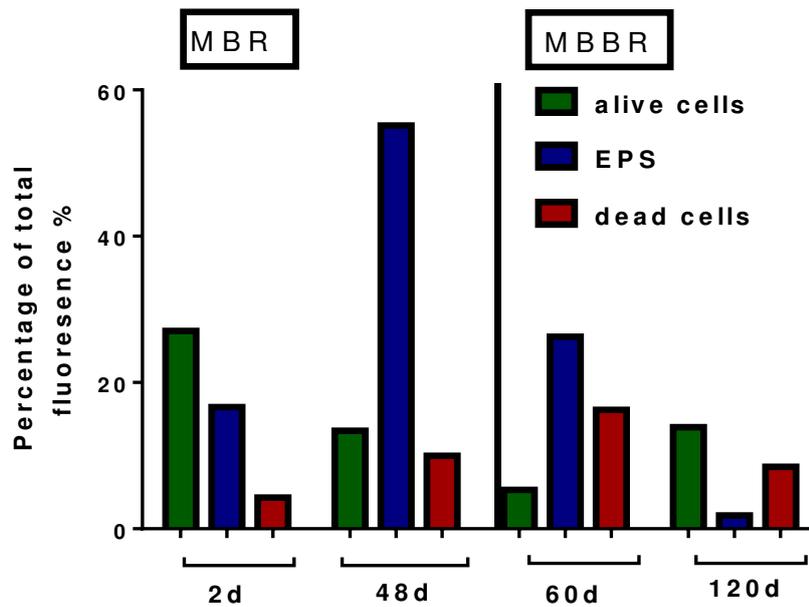
Figure 5 Distribution of EPS in the biofilm stained with Back light ™ a series of confocal images taken at same factors of realization (part [1]). (a) and (b) aerobic MBR at 2 and 48 days of treatment respectively, (c) and (d) aerobic MBBR at 2and50 (d) treatment respectively. Percentage of total fluorescence intensity (part [2]) Concentration of EPS was in Blue, dead cells in red and in Green: Concentration of live bacteria in sludge

It can be postulated that the occurrence of supports allows the development of biofilm and there by the production of EPS constitutive or sorbed on the biofilm. We also note that the

EPS concentration is not stable although we felt that we were at hydraulic steady state. This can mean that the membrane acts as a barrier especially for proteins and polysaccharides. The occurrence of EPS on the sludge and on biofilm is confirmed with the use of a confocal microscopy and the three-dimensional structure of flocs could be visualized with the marking of EPS (in blue) and viability in green). Visualization of flocs collected in the MBBR reactor and of a floc collected in BRM reactor after (2 and 50) days of exposure to pharmaceuticals compounds is presented in (Fig 5). The comparison of the evolution of the green and blue fluorescence during this period showed the increase of the EPS concentration in MBR and MBBR and that confirm our chemical analyses for the soluble EPS (bleu), but it was significantly in the MBR by compared with MBBR and that could refer to presence the biofilm supports media and mechanism of fixation the biofilms.

4. Conclusions

In this work membrane bioreactor system was operated for 120 days with reel hospital effluent. Biofilm supports media injected in the bioreactor after 60 days of experiment (MBBR). The study compared the sludge performances of MBB and MBBR, treating the same hospital wastewater, operating over a small range of solid retention time (SRT = days). The influence of biofilm supports media and separation technique (membrane) was studied and shed new light on the subject which will be useful for further development and optimization of MBR.

This study reported that:

1. The MBR and MBBR were able to achieve good organic removal efficiencies, except the VSS with the total and soluble COD. The MBBR was higher removal efficiency.

2. Tramadol sulfamethoxazole, trimethoprim, naproxen, triclosan, metoprolol, sotalol, losartan, carboxyl ibuprofen, 2-hydroxy ibuprofen, epoxy carbamazepine, 4 androstene-3, 17-dione and iomeprol could be completed removed by MBBR, while any removal by MBR was found for them.

3. The results show it found an increase of the retention of protein and polysaccharides of the sludge which depended on the attached biofilms on the supports media and, consequently the possible role of supports media in MBBR at decreasing production the proteins and polysaccharides in the soluble phase as result the fouling of membrane.

This finding confirmed that the biofilm supports media could be played a major role in increasing the efficiency of MBR system in treating the hospital wastewater.

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Upgrading the performances of ultrafiltration membrane system coupled with activated sludge reactor by addition of biofilm supports for the treatment of hospital effluents

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HIGHLIGHTS

- Biofilm supports media addition in a biological system had consequences on the global quality of treatment.
- An important improvement of pharmaceuticals removal was linked to the increase of biomass concentration.
- The occurrence of a biofilm system in a biological reactor has direct consequences on the quality of discharged effluent.
- The development of biofilm in the system permits a modification of the proportion of the major exo-polymeric substances.
- Adding a support media in the biological system will improve the functioning of the membrane.

ARTICLE INFO

ABSTRACT

The biological treatment of an hospital effluent has been monitored during 150 days in an activated sludge system followed by an ultrafiltration membrane (BBR-UF). After 75 days, support media was added into the bioreactor to develop a biofilm and to compare process performances of the two reactor configurations: activated sludge (AS-UF) or biofilm biological reactor (BBR-UF). The removal efficiencies of (chemical oxygen demand) COD, (total suspended solids) TSS, (volatiles suspended solids) VSS, and (total nitrogen) TN with the BBR-UF were 93.2%, 100%, 99.9% and 91.3%, respectively, compared to 87.9%, 99.6%, 97.5% and 91.1% with the AS-UF. Codeine, ketoprofen, diclofenac, naproxen, roxithromycin, metronidazole, hydrochlorothiazide, furosemide, gemfibrozil, pravastatin, and iohexol were highly removed by BBR-UF, while low removal was observed for the same molecules in the AS-UF. This could be attributed (1) to the increase of biomass concentration, (2) to the increase of sludge resident time or (3) to sorption on the biofilms. During continuous reactor operation, (trans membrane pressure) TMP increase in BBR-UF was negligible whereas membrane module in AS-UF required a regular physical maintenance. In the last case, membrane fouling was attributed to the modification of the concentration of the produced exopolymeric substances like protein and polysaccharide. The addition of biofilm supports media improved the performances of AS-UF and also decreased the negatives effects of the biomass on the membrane for the treatment of hospital wastewaters.

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1. Introduction

The wastewater treatment processes which combine biological treatment and membrane filtration has turned out as an attractive option for liquid solid separation combined with micropollutant removal. The membrane separation technique could be used to avoid problem of non-settling sludge, to replace a secondary clarifier, to obtain a high effluent quality and a compactness of treatment plants. In a tertiary treatment it could be used to assure a better quality of water compared to traditional activated sludge [1].

Nevertheless, fouling is one of the main drawbacks of membrane filtration because it reduces the performance of membrane over the time, and needs washing operations to overcome the occurrence of cake resistance at the membrane surface as backwashing, jet aeration, operation below critical flux, addition of

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coagulants [2]. When the fouling occurs, a thick gel layer and cake layer are formed on and into the membrane, causing the decrease of the permeate flux and the necessity to clean the membrane. Fouling is usually attributed to a number of parameters, such as sludge particle deposition, adhesion of macromolecules as extracellular polymeric substances (EPS) produced by bacteria in the biological system and pore clogging by small molecules [3]. Soluble EPS (soluble macromolecules and colloids) can enter the membrane pores, leading to a reduction of total section area of membrane pores causing pore plugging and increasing the membrane resistance [4].

Most of the studies were focused on minimizing the cake formation on the membrane surface, but another way is to use a supports media in the bioreactor to fix the biomass and thereby to limit the primary sources of cake layer and minimize the fouling [5–7]. As an example, Basu and Huck [8] studied the effect of support media in integrated biofilter submerged membrane system on membrane fouling rate and on water quality parameters. It was found an observable difference in the membrane fouling rates between the two processes and a fouling at least two times slower with the support media system compared to the non-support system. The authors also suggested that the support media enhanced the membrane surface scouring which improved the removal efficiency.

For this, the implemented process treatment consisted of a biological basin coupled to an external membrane module. After 75 days of running with planktonic biomass, plastic carriers were added to allow the development of a biofilm within the same reactor.

The objectives of this presented work, applied to the evaluation of pharmaceutical residues removal by biological treatment coupled with membrane filtration, are in a short term (i) to increase the performance of a classical AS-UF system by adding support media but also (ii) to evaluate in the same time the evolution of membrane fouling and (iii) to demonstrate, *in fine,* the relation of this physical clogging with the biochemical structure of bacterial flocs and the EPS production.

2. Materials and methods

2.1. Study area

The hospital effluent (HE) samples used in this study were collected from the sewers deserving the clinical activities of hospital (Limoges, France). The average characteristics of wastewater and those of activated sludge used as inoculum during the experiments are detailed in the Table 1.

2.2. Reactors and operating conditions

The bioreactor had a working volume of 400 L equipped with a Ruston turbine (80–120 rpm) installed to keep the bioreactor completely mixed. An ultrafiltration membrane module (ALTING, MICRODYN, France), consisting of a module of hollow fiber in polypropylene, developing 1 m² of surface area for a pore size of 0.2 μm, was positioned in an external circulation loop (Fig. 1). The influent permeate flow had an average value of 100 L/ day. The average values of hydraulic retention time (HRT) and of sludge retention time (SRT) were respectively of 22 h and 20 days, the temperature of 18–20 °C and the pH varied from 6.8 to 7.9.

Dissolved oxygen levels were automatically maintained between 1 and 4.5 mg O_2/L. The cycle of operation (feeding, pumping, decantation, filtration) was conducted and supervised by a programmed automat (AUTOMAT: market) (Table 2). Pressures were measured at the inlet ($P1$), outlet, ($P2$), and permeate side of the membrane ($P3$) in order to determine the transmembrane pressure (TMP). At constant permeate flux, TMP indicating the extent of membrane fouling was calculated as in Eq. (1):

Table 1
Physicochemical characteristics of the hospital effluents (HE) and sludge.

	COD (mg/L)		N (mg/L)		TSS (g/L)	TVSS (g/L)
	Total	Soluble	Total	Soluble		
HE	318 ± 5	185.5 ± 5	122.4 ± 4	80 ± 4	0.2 ± 0.05	0.054 ± 0.05
Sludge	1174 ± 5	144 ± 5	–	–	5.9 ± 0.05	0.121 ± 0.05
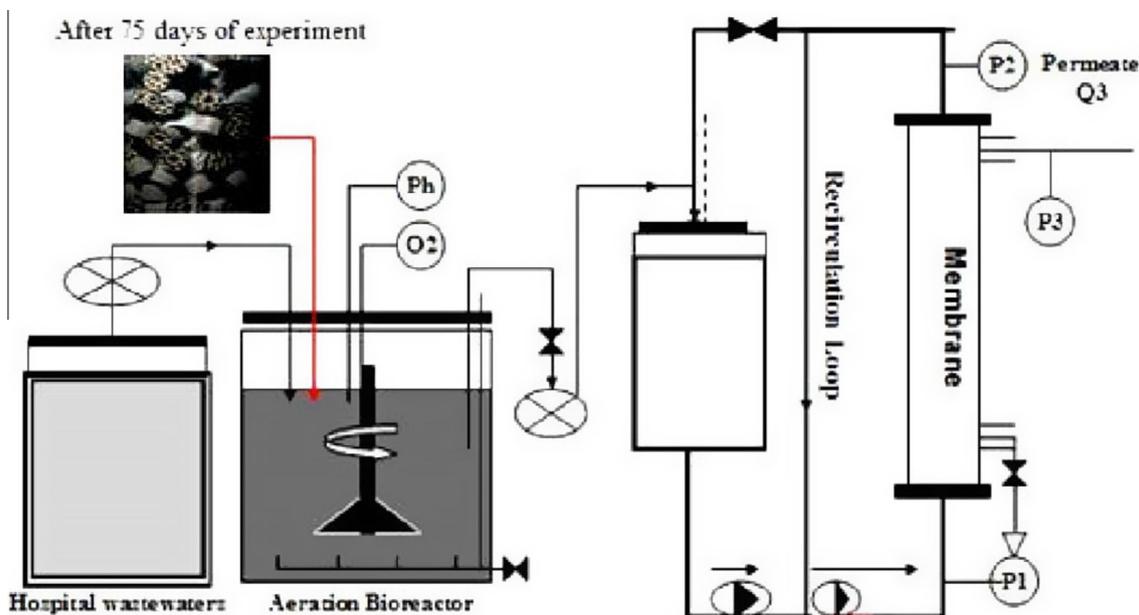
Fig. 1. Activated sludge followed by ultrafiltration system (AS-UF).

Table 2
Cycle of operation during the experiment.

Cycle of operation	
Decantation time	20 min
Transfer time	20 min
Filtration time	20 min
Feeding time	40 min
Washing- tank volume	150 L
Pump flow	900 L/h
Flow of circulation pomp	800–950 L/h
Inlet flow	4.25 L/h
TMP	0.1–0.25 bar

$$TMP = (P1 + P2)/(2 - P3) \tag{1}$$

After 75 days of operation by the activated sludge coupled with the membrane (AS-UF), biofilms supports media (Christian Stöhr, Germany) were added to the bioreactor to begin the second period of treatment (BBR-UF). The supports are made of HDPE (high density polyethylene) and have the following characteristics: a specific surface area of 600 m^2/m^3, diameter of 12.2 mm or a length of 12 mm, density of between 0.95 and 0.98 and weight of 150 kg/m^2.

2.3. Analytical methods

The characteristics of wastewater and sludge were assessed every two days. Total and volatile suspended solids (TSS and VSS) were measured according to the normalized method [10]. The chemical oxygen demand (COD) and total nitrogen (TN) were determined on both total and soluble fractions (filtration at 0.45 μm) by micro-methods as instructions of HACH methods (HCT 191, ISO 15705 and HACH DR/2000).

Two different analytical methods were applied by IANESCO laboratory, (France) to determine the concentration levels of the pharmaceuticals in the wastewaters samples. Water samples were enriched by liquid–solid phase (SPE) by using Oasis HLB cartridges (6 ml, 0.2 g) from waters. The SPE extracts were injected in liquid chromatography mass spectrometry (LC–MS/MS) applying electrospray ionization (ESI) under high-resolution MS conditions. Quality control (QC) was assured by measuring two transitions for each analyze and each internal standard, comparing retention time of analyze with the retention time of the internal standard in each sample, duplicates, numerous blanks, and QC standards. The global analytical error was ±0.75 μg/L.

The global removal efficiencies were determined according to Eq. (2):

$$Removal\% = 100 \times [(C1 - C2)/C1] \tag{2}$$

with C_1: experimental concentration in the influent (input) and C_2: experimental concentration in the effluent (output).

The mass balance was determined according to Eq. (3):

$$Q_{in}[EPS]_{in} + RV = Q_{out}[EPS]_{out} + \frac{d([EPS]_{out}V)}{dt} \tag{3}$$

with Q_{in} and Q_{out} influent and effluent flow rate (L/d).
    R: EPS production rate (mg/L D).
    [EPS]: concentration of the considered EPS (mg/L) in the input or output.
    V: volume (L).

2.4. Confocal laser scanning microscopy, EPS staining and digital images analysis

Polysaccharides (PS) and proteins (PN) staining was carried out according to the modified procedure of [11]. Samples of mixed liquor were centrifuged to remove supernatant, washed twice with

1× phosphate-buffered saline (PBS) buffer (pH 7.2) and kept fully hydrated in 2 mL centrifuge tubes covered with aluminium foil. For PS staining, 100 μL of concanavalin-A conjugated with tetramethylrhodamine (Con A, 250 mg L^{-1}, Molecular Probes, and Carlsbad, CA, USA) was first added dropwise to the sample and incubated for 30 min to stain α-mannopyranosyl and α-glucopyranosyl sugar residues. For PN staining, 100 μL of sodium bicarbonate buffer (0.1 M) was introduced to the sample to maintain the amine groups in non-protonated form. Subsequently, 100 μL of fluorescein isothiocyanate solution (FITC, 1 g L^{-1}, Fluka) was supplemented and incubated for 1 h to bind to proteins. Samples were washed two times with 1× PBS buffer (phosphate buffered saline) after each staining stage to remove loosely bound and excess dyes. Finally, sectioned granule or biofloc samples were positioned onto microscopic glass slides for observation of the distribution of PS and PN by a confocal laser scanning microscopy equipped with an Ar–He–Ne laser unit and three barrier filters. The image acquisition settings, such as laser intensity, numerical aperture, gain and offset settings were adjusted according to [12–14] and the levels were kept constant through observation. Samples were visualized with a ×10 objective and analyzed with the start LSM image browser confocal software and Image J software.

Image analysis was performed with the freely available software Image J version 1.39i including the LSM Reader plug into open LSM5 formatted image stacks created by the microscope software. The tool Image J Analyzer 1.1, which is based on the performance of Image J and handles LSM5 formatted image stacks, was programmed for quantitative analysis. By setting a threshold, pixels with intensity below the threshold were assigned to the background. All other pixels were set to the foreground. Due to the individual image adjustment during the image stack acquisition, the threshold was chosen manually for each image stack. The settings were made according [12,15].

2.5. Characterization of the biofilm

Various methods have been implemented to understand the effect of biochemical water quality on membrane filtration performances. As it is known, the biopolymers produced by the cells (EPS) or present in the wastewater could have an impact of membrane clogging [16]. Thus, the growth of biofilm has been monitored and total EPS estimated by classical biochemical analyses and by CLSM image analyses after staining (polysaccharides (PS), proteins (PN), humic like substances (HA)) as illustrated Fig. 2.

The concentration of attached biomasses on the supports was estimated by Eq. (4) according to [17]:

$$Y = (Vbio.\rho bio)/(Vc.\rho c) \tag{4}$$

where Y is fraction of attached biomasses in (mg biofilm/g PVC), $Vbio$ is the volume of biofilm in (m^3), (ρbio) is the density of biofilms (kg/m^3), Vc is the volume of supports media (m^3) and (ρc)is the density of the supports media in (kg/m^3).

3. Results and discussion

3.1. Treatment of pollution and pharmaceuticals removal from hospital effluent

The treatment of an hospital effluent has been running during 150 days, with an operating cycle without biofilm supports during the first of 75 days (AS-UF) and the addition of these supports the 76th day (BBR-UF).

Both wastewaters systems exhibited high rates of organics compound removal, however lower values in term of total and soluble COD, TSS and VSS removal were observed in the (AS-UF) com-
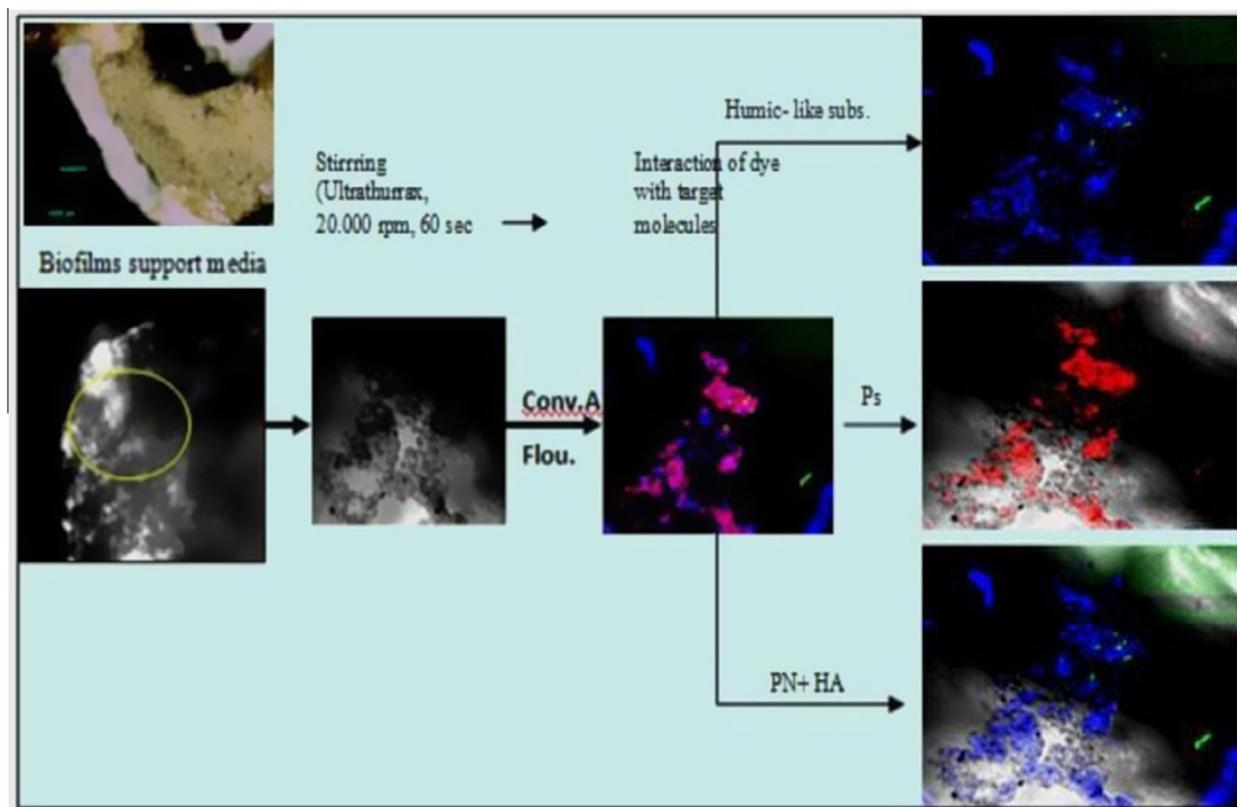
Fig. 2. Microscopic Images represent the biofilms fixed on supports with the time. (B) CLSM images of the EPS distribution within biofilms attached on the supports media. Protein was in (green) and ConcA staining α-mannopyranosyl (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pare to the BBR-UF (Table 3). As described previously by [8,9] these results demonstrated that the presence of supports media allowed an increase of global microbial activity due to the increase of biomass concentration on the support and of the SRT of fixed organisms.

The concentrations of the analysed pharmaceutical compounds and some of their transformation products during the operation period were determined by LC/MS–MS in the permeate influent and in the sludge. (Fig. 3) illustrates the concentration of pharmaceuticals in influent and permeate at steady state after 75 days (AS-UF) and after 150 days of the operation (BBR-UF period). A highest removal efficiency (≈95 ± 5%) of codeine, pravastatin, ketoprofen, diclofenac, roxithromycin, gemfibrozil, and iohexol were observed in BBR-UF compared to AS-UF system in which low or no removal was achieved. As an example, ketoprofen was eliminated by 62% in AS-UF and by to 97% in BBR-UF probably due to addition the biofilm supports media.

Kathryn et al. [18], were already demonstrated the role of the biofilm in increasing efficiency of the removal of pharmaceuticals in laboratory tests. For example, the removal of diclofenac increased to from 0% in AS-UF to 30% after 60 days with supports media. Falas et al. [19] indicated that diclofenac and clofibic acid were not removed in an activated sludge reactor, while they were in a carriers reactor. Theses same authors, [20] in a more recent paper, confirmed that some micropollutants, as diclofenac or tri-

methoprim, showed significantly higher removal rates with biological system of treatment with carrier than without. They explained that high sludge ages could favour degradation of some pharmaceuticals. They suggested also that a microbial adaptation to the substrate gradients in biofilm could increase their degradation. These results confirm those of Clara [21] about the importance of the SRT control for the removal of recalcitrant pharmaceuticals.

The gemfibrozil, compound with a complex chemical structure, was also analysed in the sludge of the AS-UF and of the BBR-UF; it was detected only in the BBR-UF system. This could be attributed to the increase of the biofilm concentration in the reactor, and then, to sorption phenomenon. This confirms the results of [22] for ketoprofen, finding that this molecule was not eliminated in an AS treatment process.

Refs. [18,21–23,24,25] confirmed that ibuprofen exhibits high value of biodegradation kinetic coefficient in range of 20, 9–35, l g SS^{-1} day^{-1}. The hydrophilic nature of this substance makes its sorption onto sludge negligible, which means that the main removal mechanism of ibuprofen is due to a biological degradation. A high removal efficiency of ibuprofen (>90%) was reported, according with our study with respectively 95% in AS-UF and 96.4% for BBR-UF.

3.2. Impact of biofilm support addition on membrane performance

Fig. 4 showed the changes in biomass concentration in the bioreactor and in the outlet of bioreactor before and after introduction of the biofilm supports media in bioreactor after 75 days of AS-UF. Before the introduction of supports, the TSS in the reactor was globally constant (1500 mg/L) showing that the system was at the steady state, but the concentration at the outlet was very noisy and unstable. This could have some consequences on the mem-

Table 3
Stabilized COD, N and TSS removal efficiencies for AG-MBR and SG-MBR.

Removal Efficiency (%)	TSS	VSS	Total		Soluble	
			COD	N	COD	N
AS-UF	99.6	97.5	87.9	91.1	86.9	90.5
BBR-UF	100	99.9	93.2	91.3	91.8	90.8

PPCPs	Influent (µg/L)	Outlet (µg/L)	
		AS-UF	BBR-UF
Iohexol	167	128	0.334
Iopromide	1.1	<DL	0.2805
Carboxy-ibuprofen	16	4.14	<DL
Fenofibric Acid	2.1	<DL	0.5901
Atenolol	0.77	<DL	0.23023
Metroprolol	0.1	<DL	0.01
Pravastatin	0.42	0.1665	<DL
Gemfibrozil	0.12	0.8	<DL
caffeine	41	<DL	1.599
Furosemide	5.1	1.521	1.3005
Hydrochlorothiazide	2.8	2.1	2.5004
Trimethoprim	1.4	0.032	1.1998
Metronidazole	4.8	3.417	2.0976
sulphametoxazole	1.6	0.084	0
Roxithromycin	0.21	0.36	0.09009
Ibuprofen	1.4	0.045	0.0504
Naproxen	3.7	0.52	0.1998
Diclofenac	0.1	0.25	0.07
Paracetamol	177	<DL	<DL
Ketoprofen	6.4	2.242	0.192
Codeine	0.18	0.11	<DL
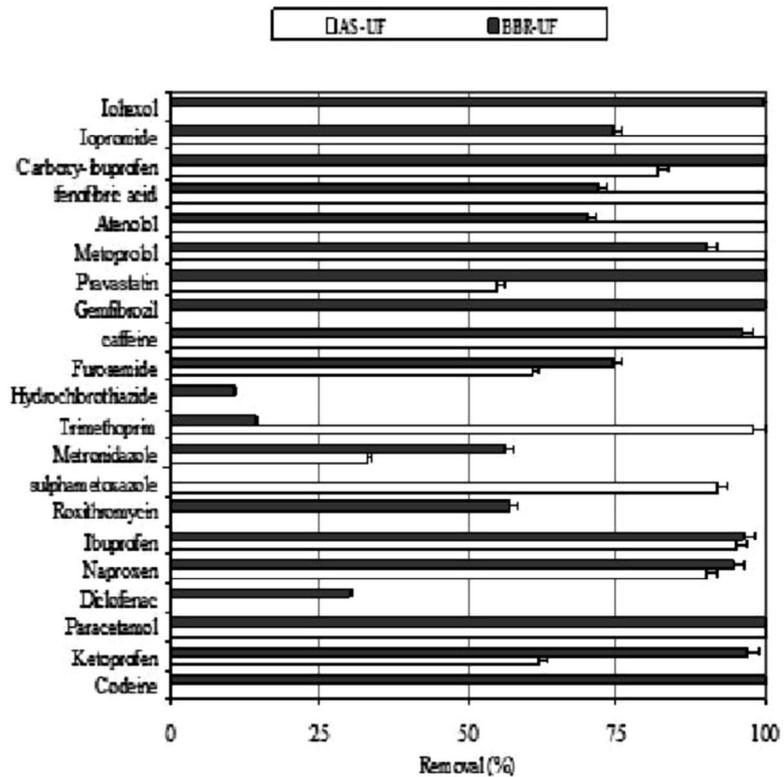
Fig. 3. Concentrations and removal rates of PPCPs in both AS-UF and BBR-UF systems.
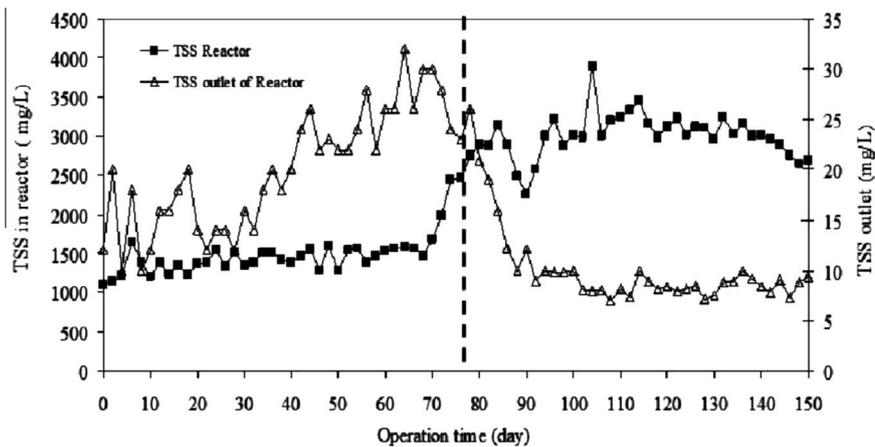
Fig. 4. TSS (mg/L) in the bioreactor and in the outlet before and after introduction the biofilm supports media.

brane filtration system operation. After the addition of supports, the TSS concentration (Biofilm + free cells) doubled to a stable concentration of 3000 mg/L while the TSS concentration at the outlet decreased to a stable concentration of 10 mg/L.

Because an increase of TSS in the discharged water could have some consequences on the quality of the filtration due to the membrane fouling, the TMP was measured. Fig. 5 showed that in AS-UF system, TMP was maintained around 15–25 kPa during 75 days of continuous reactor operation (0–75 days). On day 75, when biofilms supports media were added in BBR-UF, the TMP was reduced to reach about 17 kpa indicating a restoration of membrane permeability and a stabilization of the flux around 50 L h^{-1}. This fact was observed without the use of chemical washing. This result suggests that the presence of support media notably improved the membrane performances.

3.3. Biofilm growth and EPS characterisation and localisation after support media addition

Fig. 6 showed distinctly the increase of thickness of biofilms and the concentration of attached biomasses on the supports estimated according Eq. (3).

After 110 days of experiment the average thicknesses in middle of the total biofilm on supports was 400 µm measured by bifocal inversed microscopy (STEMi V6 coupled with software Videomet).

Images of confocal microscopy of biofilms fixed on the supports media after staining confirm the occurrence of EPS in biofilm.

Soluble and total EPS were represented in the Fig. 7a and b. The total and soluble EPS concentrations, their composition (PN, PS, HA) and their evolutions by biochemical analyses and microscopic techniques with fluorescent staining were determined during the
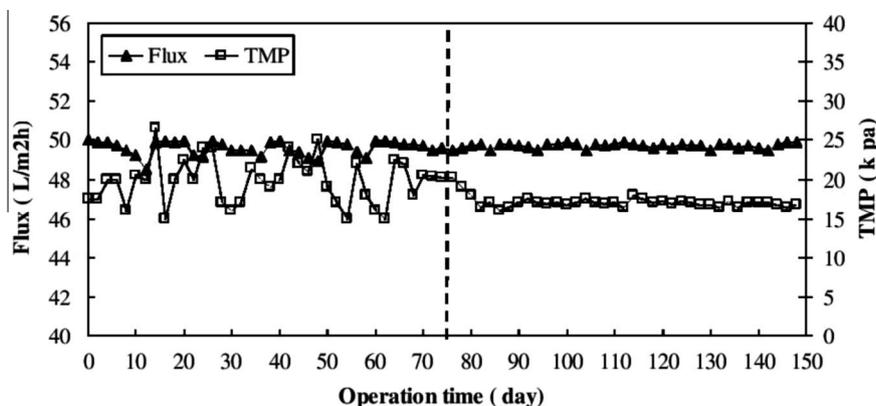
Fig. 5. Transmembrane pressures and permeate flux of BBR-UF and classical MBR as a function of operation time.
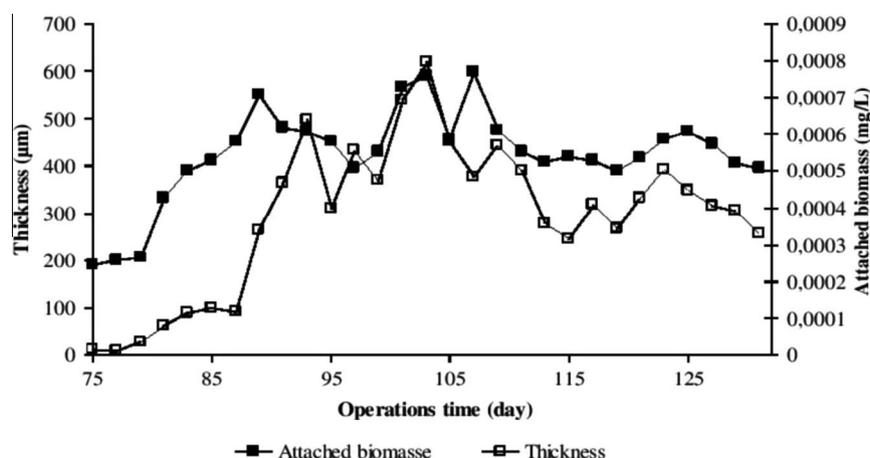
Fig. 6. Evolution of the thickness and attached biomasses on supports media versus time.

150 days of operating illustrated in Fig. 8. Significant difference could be found between the first period (before 75 days) and the second period (after 75 days) of operation.

Increasing concentrations of PN, PS, HA in both total and soluble phases was observed in the 20 first days of operation followed by a decreased of the PN, PS and HA concentrations to reach about 25, 15 and 180 mg/L, respectively for the total phase and 10, 8 and 148 mg/L, respectively for the soluble phase. The evolution of these concentrations could be due to the biomass acclimation to the hospital effluent, to a bacterial reaction against the occurrence of pharmaceuticals compounds in the effluent [26] or, for a part, directly by a certain quantity of EPS brought in by the influent (see after).

After 75 days and the adding of supports, these concentrations of PN, PS, HA were globally constant to reach the values of 5–10 mg/L, 30–45 mg/L and 160–220 mg/L, respectively for the total phase and 3–8 mg/L, 10–20 mg/L and 175 mg/L, respectively for the soluble phase.

3.4. Explanation of membrane clogging improvement trough EPS mass balance

The occurrence of biofilms increases the concentration of EPS, which are intrinsic of their structure. Therefore, their concentrations in the supernatant depend on their adsorption onto microbial flocs, their removal by sludge clogging and their passage through the membrane [27]. Presence of the biofilm supports media in the sludge was believed to play a significant role at accumulation and absorbing the biofilm and consequently, changes the concentration of EPS in the reactor. To verify the influence of the quality of wastewater on the occurrence of EPS in the system, a mass balance between input and output has been done (Eq. (3)) considering the concentration of PS, PN and HA in the input and a average flow rate.

The result confirmed a production per day of EPS, especially for PS and HA with 20 mg/d and 250 mg/d respectively. The results showed that water quality had a minor influence compare to the EPS production by the microorganisms.

The effect of the enhancement of PS and HA, and especially the decrease of PN is directly correlated with the improvement of membrane filtration because it is now well known that the concentration of a protein was one of the reason of membrane clogging and fouling as shown in [27].

The evolution of the EPS fluorescence during this period showed the decrease of proteins, the increase of polysaccharides and relative stability of the humic-like substances after the addition of support media (Fig. 9). These observations were in agreement with our chemicals analyzes for the EPS compounds during the experiment. It confirmed the importance of EPS, especially proteins, in the biofouling phenomena, and the possibility of the control of the efficiency of a membrane system by a biological-based strategy, as suggested by [28].
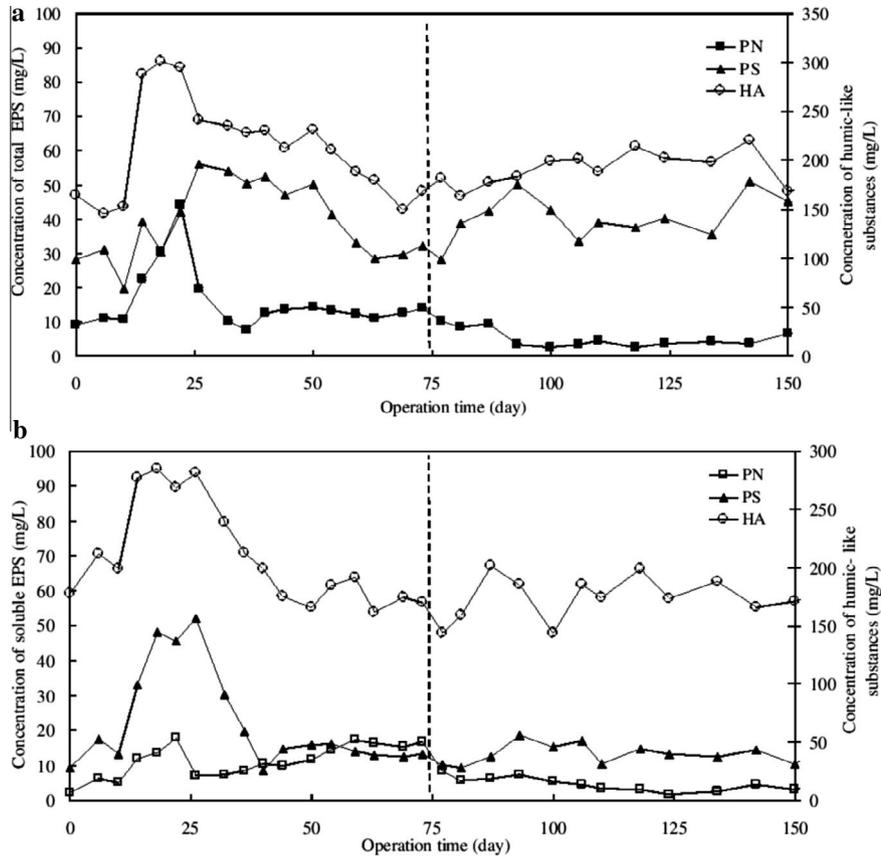
Fig. 7. Variation of concentration of total EPS (a) and soluble EPS (b) in versus of operations time (day).
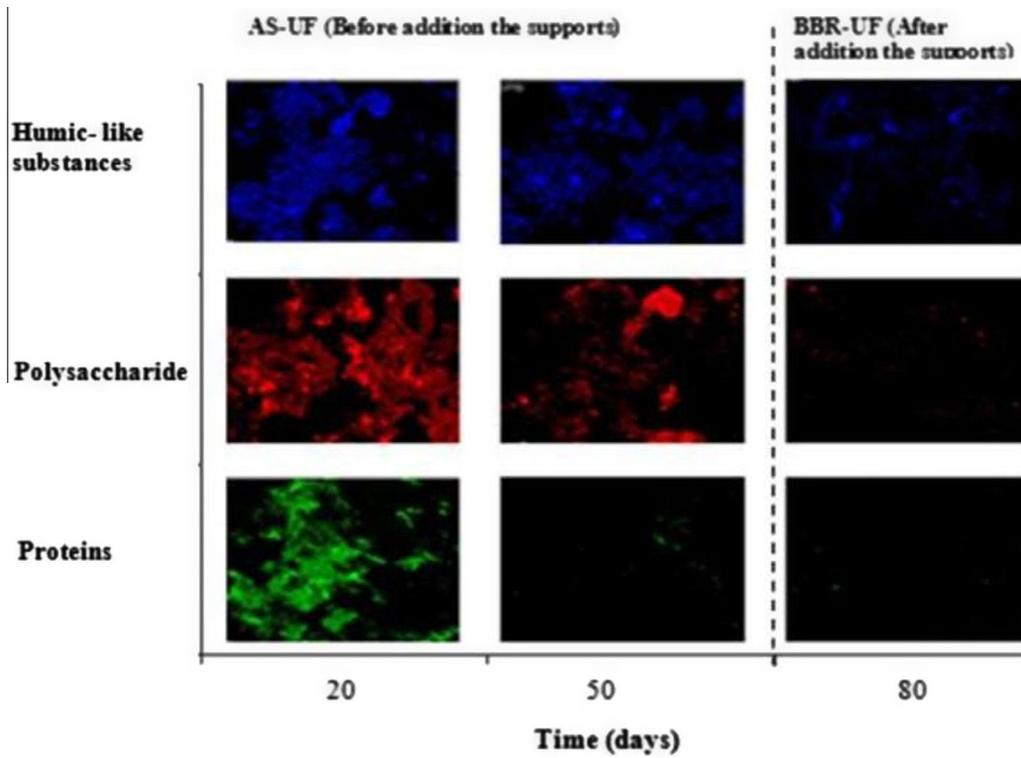
Fig. 8. CLSM images of the EPS distribution within AS-UF and BBR-UF flocs. Images were obtained at $10\times$ magnification.
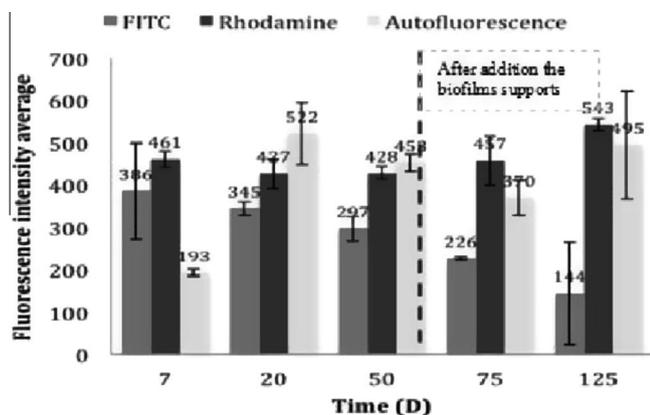
Fig. 9. Average fluorescence intensity in different periods of operation statistical analyses of the *Z* stack analysis by image J (three-dimensional structure).

4. Conclusions

Biofilm supports media addition in a biological system followed by ultrafiltration membrane had consequences on the global quality of the treatment with a slight increase of performance (the removal efficiencies of COD, TSS, VSS, and TN with the BBR-UF were 93.2%, 100%, 99.9% and 91.3%, respectively, compared to 87.9%, 99.6%, 97.5% and 91.1% with the AS-UF), coupled to an important improvement of pharmaceuticals removal ($\approx 95 \pm 5\%$) for pravastatin, ketoprofen, diclofenac, roxithromycin, gemfibrozil, codeine, and Iohexol. This result was linked to the increase of biomass concentration, of the solid resident time and of the sorption capacity. This membrane efficiency is function of the fouling phenomena, dependant of the quality of the influent and by washing operations. The occurrence of a biofilm system in a biological reactor has direct consequences on the quality of discharged effluent, retaining the suspended solid in the biological reactor and protecting the membrane. It was shown in this study that the development of biofilm in the system permits a modification of the proportion of the major exo-polymeric substances in the soluble phase compared to a free cells system. The concentration of proteins, identified as a cause of clogging in membrane system, decreases which induces a better stability of the transmembrane pressure. In conclusion, adding a membrane system to a biological free cells treatment will improve the quality of the effluent, and adding a support media in the biological system will improve the functioning of the membrane; consequentially, the decrease of operating cost could compensate the equipment cost.

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Efficacité du charbon actif en grain modifié couplé à un bioréacteur à membrane pour le traitement de micropolluant organique

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Ce travail part toujours du principe de l'amélioration de l'élimination des composés pharmaceutiques des effluents hospitaliers. La configuration choisie dans ce cas est le système à boue activée, suivi d'une membrane d'ultrafiltration, suivi par une colonne de charbon actif en grain, dont l'objectif est l'élimination des polluants résiduels.

La colonne de CAG a été divisée en 3 parties, et le charbon traité différemment dans chacune d'elle (lavage acide, sans lavage, et lavage basique) dans l'objectif de modifier les propriétés du charbon et ainsi de capter le maximum de molécules, malgré leurs différences de propriétés physico-chimiques.

Comme dans les cas précédent, et lors de 275 jours de traitement, les résultats d'épuration mesurés sur les paramètres classiques sont très bons.

Les analyses des résidus médicamenteux ont portés sur 21 composés pharmaceutiques, dans l'effluent, en sortie de réacteur membranaire, et suite aux colonnes de GAC modifié. Certains composés sont bien éliminés par le traitement biologique membranaire (ketoprofène, naproxène, paracétamol, ibuprofène, caféine, gemfibrozil, pravastatin, carboxyl-ibuprofène, iohéxol). Les premiers résultats du couplage MBR – GAC ont montré une élimination proche de 100% sur les différentes molécules analysées résiduels.

Article 6

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Chapter III

Efficiency of Modified Granular Activated Carbon Coupled with Membrane Bioreactor for Trace Organic Contaminants Removal

Mousaab Alrhmoun, Magali Casellas, Michel Baudu, Christophe Dagot

Abstract—The aim of the study is to improve removal of trace organic contaminants dissolved in activated sludge by the process of filtration with membrane bioreactor combined with modified activated carbon, for a maximum removal of organic compounds characterized by low molecular weight. Special treatment was conducted in laboratory on activated carbon. Tow reaction parameters: the pH of aqueous middle and the type of granular activated carbon were very important to improve the removal and to motivate the electrostatic Interactions of organic compounds with modified activated carbon in addition to physical adsorption, ligand exchange or complexation on the surface activated carbon. The results indicate that modified activated carbon has a strong impact in removal 21 of organic contaminants and in percentage of 100% of the process.

Keywords—Activated carbon, organic contaminants, Membrane bioreactor.

I. INTRODUCTION

IN the world, the question of presence the micropolluant as pharmaceuticals and personal care products (PPCPs) in water is one of main problems of environment, because of sanitary and dangerous consequences for this type of micropolluant and the insufficiency of purification networks. Hospitals are important sources of these compounds: a great variety of micro-contaminants result from diagnostic, laboratory and research activities on one side and medicine excretion by patients on the other. They include active principles of drugs and their metabolites, chemicals, heavy metals, disinfectants and specific detergents for endoscopes and other instruments, radioactive markers and iodinated contrast media [1], [2]. In France, the total number of hospitals has raised from 1540 in 1990 to 2856 in 2005. Environ 1.071.000 m3/d hospital wastewater was generated, corresponding to approximately 5 % of municipal wastewater in 2005.

The Membrane Bioreactor (MBR) technique is a promising alternative to conventional treatment as membranes can achieve a high degree of water purification. The combination

Of membrane filtration and biological treatment avoids secondary clarification and tertiary steps [3]. Recently, more attention has been paid to the membrane bioreactor (MBR) technology for hospital wastewater treatment because of its

higher efficiency in pollutant removal, excellent effluent quality, low sludge production, compact size and lower energy consumption [3]. Because of their ability to reach higher contact times and then to maintain in reaction a slow-growing biomass, microorganism species are more diversified with higher physiological capacity and are more adapted to resistant compounds. Although the effectiveness of MBR treatment for eliminating trace organic contaminants has been well demonstrated in the literature, recent studies have also shown the limitations of MBR in removing certain persistent compounds [4]-[6]. Therefore, it is necessary to implement a post-treatment process after MBR particularly in indirect potable water recycling applications or when discharging the effluent to an ecologically sensitive environment.

Numerous authors have investigated the MBR for the treatment of effluent containing pharmaceuticals [5], [2]. All these studies were carried out using microfiltration (MF) or ultra filtration (UF) membranes. In this study, the removal of trace organic contaminants via sequential application of GAC adsorption following MBR treatment (MBR-GAC) was investigated. Using the granular activated carbon (GAC) adsorption has been commonly in treatment process of industrial water and it is very effective for the removal of pesticides and other emerging trace organic contaminants in drinking water treatment [7], [8]. Recently, a few have investigated the use of GAC adsorption for the removal of trace organic micropolluant from biologically treated effluent [9]-[11]. The hospital wastewater treated by membrane reach to GAC post-treatment which to specifically target the residual trace organic contaminants in MBR permeate.

The purpose of this paper was to summarize the long-term performance experience of a MBR- GAC system for hospital wastewater treatment and to provide data on the elimination efficiency of an on-site biological wastewater treatment. To verifier this objective: (i) pilot-scale MBR coupled with post treatment GAC was installed to receive and treat real hospital wastewater, (ii) an efficient and representative samples was taken to representatively collect influent and effluent from the MBR; and (iii) SPE-HPLC-MS/MS analytical method was developed and optimized to quantify the concentrations of approximately 30 target analytics including pharmaceuticals and human metabolites (laboratory INASCO, Poitiers, France).

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II. MATERIAL AND METHODS

A. Study Area

The hospital effluent (HE) samples used in this study were collected from the sewerage system which comprises only sewers from clinical activities of the hospital. Average characteristics of wastewater and activated sludge used as inoculums during the experiments are detailed in Table I.

TABLE I
PHYSICOCHEMICAL CHARACTERISTICS OF THE HOSPITAL EFFLUENT (HE),
AND ACTIVATED SLUDGE (AS)

| | COD (mg/L) | | N (mg/L) | | SM (g/L) | VM (g/L) |
	Total	Soluble	Total	Soluble		
HE	333.801	177	128	84	0.1965	0.061
AS	1201	145	-	-	7.25	1.52

B. Lab-Scale MBR and GAC Post-Treatment Column

The reactor consisted of a membrane bioreactor with a working volume of 400 L and a membrane module in an external circulation loop. The membrane module was a polypropylene and type of fibers creuses (MF) membrane with 1m² of surface area and pore size of 0.2μm (ALTING, MICRODYN, France) (Fig. 1). A Ruston turbine (80-120 rpm) was installed to keep the bioreactor completely mixed. An identical lab-scale cross-flow MBR was run and inoculated with activated sludge from a municipal wastewater treatment plant (dry weight, 2.5 g/L). The influent was a hospital effluent (average flux 100 L/day).
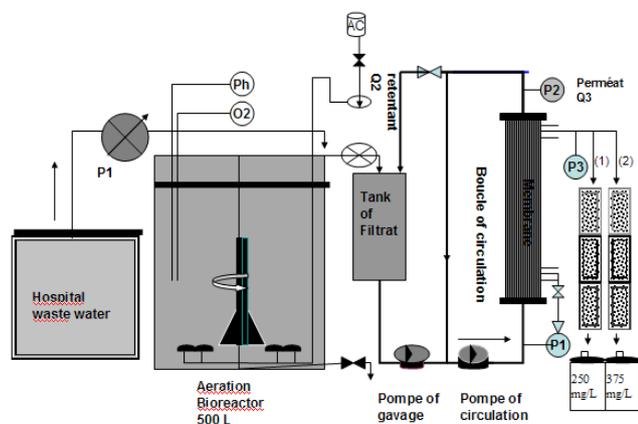
Fig. 1 Schematic diagram of the membrane bioreactors

Table II shows all the operational conditions. Daily monitoring revealed that the pH of the mixed liquor was in the range of 7.3e7.5. The aeration cycle was automatic based on tow limits. Pressures were measured at the inlet (P1), outlet (P2), and permeate side of the membrane (P3) in order to determine the trans-membrane pressure (TMP). At constant permeate flux, TMP indicates the extent of membrane fouling and it was calculated as follows:

$$TMP = [(P1 + P2) /2 - P3] \qquad (1)$$

TABLE II
KEY OPERATIONAL PARAMETERS OF MBR SYSTEMS INVESTIGATED

Condition of bioreactor operation	
Operating parameters	Operating range
Concentration of oxygen	1- 5 mg O2/L
PH	6.9 -8
T C°	14.5- 20
Agitation	80-120 tr/min
Volume (L)	400 L
flow of outlet (L.d-1)	1300-1700 L / j
SRT (d)	15-20 days
HST	22 h
Aeration	Auto - 1-5 mg.O2/L
Time of presence O₂ (h)	6
Flux outlet (14-20°C) (L.m-2.h-1)	40-50 L/h
Mode of filtration	position horizontal
Tangential speed along the membrane (m / s)	0.286m/s
Type of treatment	Decantation
Cycle of operation	
Time of decantation	20 min
Time of transport	20 min
Temps of filtration	20 min
Temps of alimentation	40 min
Volume of tank	150 L
Volume of tank the washing	150 L
Flow of pump Booster	900 L/h
flow of pomp of circulation	800-950 L/h
flow of inlet	4,25 L/ h
TMP	0,1 - 0,25 bar

The MBR permeate was further treated by a laboratory scale GAC column. The GAC adsorbent (GAC-1240) was supplied by ''Norit Activated Carbon''. The physical and chemical characteristics of this GAC are summarized in Table III and Fig. 2.

Prior to the experiment, the GAC was washed with distilled water to remove fine particles and then dried at 105C° for 24h. Two columns of borosilicate glass with internal diameter of 5cm and active length of 75cm were used in this study. The first column was filled with activated carbon in concentration 250gr of GAC/ L and the second in concentration 375gr of GAC/ L. However, the quantity was devised to three equals' parts the first part was washed by HCL (1N) in concentration 30% for 2h then dried at 30°C for 24h with pH = 4.5. The third part was washed by NaOH (1N) in concentration 30% for 2 h then dried at 30°C for 24h with Ph= 9 then the second without any treatment.
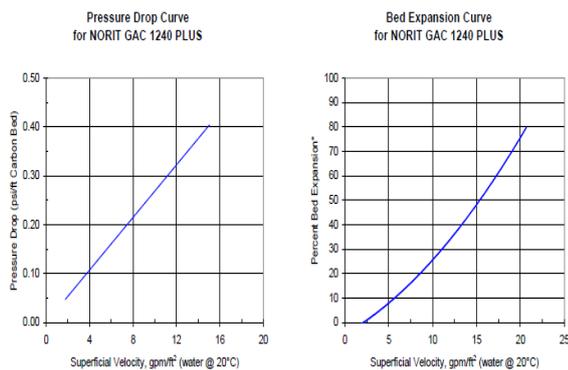
Fig. 2 Represented the pressure Drop Curve and the bed expansion curve for GAC1240 plus

TABLE III
CHARACTERISTICS OF THE GAC-1240 PLUS

Parameter	Values
Iodine number	mg/g 950 min.
Molasses number	210 min.
Abrasion number (AWWA)	78 min.
Iron, acid soluble, % as Fe	0.01 max.
Acid soluble ash, %	0.5 max.
Moisture, % as packed	3 max.
pH, water extract	5.0 to 8.0
Mesh size (U.S. Sieve Series)	
Greater than 10 mesh (2.00 mm),	5 max.
Less than 40 mesh (0.42 mm),	0.5 max.
Pore volume (cc/g)	0.046b
Pore diameter (nm)	3.232b

All the parts supplied by the columns in order and the same operation repeated for the second columns with concentration 375gr of GAC /L. (Fig. 1). This study was conducted over total 275 days, with 85 days of MBR-only operation, 145 days of operation in MBR+ support media mode and 45 days of MBR- GAC operation.

C. Analytical Methods

Wastewaters and sludge physic-chemical characteristic measurements were done every two day. Measurements of total and volatile suspended solids (TSS and VSS) were done according to the normalized method (AFNOR, NF T 90-105). Chemical Oxygen Demand (COD) was measured by the closed reflux colorimetric method (ISO 15705:2002), and total nitrogen (TN) was assessed using the alkaline per sulfate digestion with colorimetric reactive (Hatch company). The COD and TN were carried out on both total and soluble fraction (after samples filtrated at 1.2µm). Ionic species in solution were determined on samples filtrated at 0.22µm using ion chromatography (DIONEX 120) according to the standard method (AFNOR, NF EN ISO 10304-1). The used detector was conducted metric, and the analytical error was ±5%.

D. Sampling

A peristaltic pump was used for sampling of the pilot plant influent. Fresh MBR-effluent was sampled continuously by a peristaltic pump before it entered the MBR permeate tank. The flow from the sampling pumps was directed into cooled glass bottles located in a refrigerator at 4°C. Cooling elements were used during sample transport from the pilot plant to the lab for the analyses. Three sampling campaigns took place for Inlet, outlet of BRM and outlet of GAC post a preliminary over 5 weeks in June 8, 2013.

E. Sample Preparation

The wastewater samples were filtered through a 0.7-µm GF/F glass−fiber filter (Whatman, Dassel, Germany) and further through a 0.2-µm regenerated cellulose filter (Sartorius AG, Gotingen, Germany). For the analysis of 52 micro pollutants, samples were diluted ratio 1:100 and 1:10 with nano-pure water or left undiluted, depending on the matrix. Subsequently, 50 isotope labeled internal standards in three mixtures were spiked. Prepared samples were stored at 4°C in the dark for 1−20 days before they were analyzed. For analysis, 20mL of the filtered and internal standard containing sample in an amber glass vial was inserted into a cooled auto sampler rack, and automatically acidified by formic acid (0.1% formic acid in a sample, v/v) just before injection into the online SPE-HPLC- MS/MS system to avoid hydrolysis.

F. Analysis of Trace Organic Contaminants (PPCPs)

Two different analytical methods were applied to determine the concentration levels of the PPCPs in the wastewaters samples. Analyses were performed by the IANESCO. Water samples were enriched by liquid-solid phase (SPE) by using Osis HLB cartridges (6ml, 200mg) from waters. The SPE extracts were injected in liquid chromatography- mass spectrometry (LC-MS/MS). Acquisition was performed in selected reaction monitoring (SRM) mode and tow transitions (quantification, confirmation) were obtained for each compound. Quality control (QC) was assured by measuring two transitions for each analytic and each internal standard, comparing retention time of an analytic with the retention time of the internal standard in each sample, duplicates, numerous blanks, and QC standards.

III. RESULTS AND DISCUSSION

A. The Process Performances

The total and soluble COD removal efficiency was always respectively greater than 87.9 % and 86.9. During start-up TSS and VSS concentrations in the MBR increased almost continuously (depending on our wastewater characteristics the increased was slowly and not very remarkable). Effluent solids concentrations were always very low (<0.0012 g/L) confirming the excellent solids removal of micro-filtration systems. The removal of TSS was 99.5% obtained only by the filtration by membrane and that indicate to the perfect solids retention capacity of the membranes. By the way, more than 97% of the VSS influent was removed. Particular attention has to be paid to the nitrogen removal efficiencies. The total and soluble Nitrogen removal efficiency was always respectively greater than 91% and 90%. It is not worth to confirm that the denitrification potential of a wastewater is linked not only to the COD availability in the influent but also to its ready

biodegradability. Performance of the MBR with respect to the removal of TOC and TN was stable during the entire study (Table IV).

TABLE IV
EVOLUTION THE EFFICIENCY REMOVAL OF ORGANIC POLLUTANTS BY MBR

Removal %	TSS	VSS	TCOD	SCOD	TN	SN
MBR	99.5	97.4	87.9	86.9	91	90.4

B. Removal of Trace Organic Contaminants by MBR

The main mechanisms responsible for the removal of pharmaceutical compounds in the MBR-system are sludge sorption and biodegradation by microorganisms present in the wastewater [12]. The total removal efficiency (sludge sorption+ biodegradation + membrane retention) of each pharmaceutical compound was determined MBR according to (1):

$$\text{Removal \%} = (C2-C1) / C2 \times 100$$

where:

C1: the experimental concentration determined for each pharmaceutical compound in each reactor influent by LC / (MS MS) analysis.

C2: the experimental concentration of each pharmaceutical compound in each reactor effluent by the LC / (MS-MS) analysis.

The concentrations of the various pharmaceutical compounds and their transformation products during the spiking period were determined by LC/MS-MS applying electro spray ionization (ESI) under high resolution MS conditions. Table V shows the concentration of the PPCPs in influent, outlet and the removal % for the MBR after 275 days of the operation. It can be clearly observed the highest removal efficiency (environ 95%) or complete removal of Ketoprofen, Naproxen, Paracetamol, Ibuprofen, Caffeine, Gemfibrozil, Pravastatin, Carboxy-ibuprofen, Iohexol in BRM for the compounds which (log D< 3).

In our study after 275 days of operation and in high efficiency of removal for the N and the COD we can confirm that the BRM was reached to stable operation. Therefore, the high and/or variable removal of these compounds can be attributed to their physicochemical proprieties as the

electronics strong in presence functional groups (amide, chlorine and carboxylic) [6], (see Fig. 3). In addition to that effect of hydrophobic interactions and its changes with the time. Hydrophpic compounds (log D> 3) adsorbed on sludge can be retained by membrane and further biodegradation by biomasses in the reactor can occur. The reporters [4]-[6] confirmed our results. In another side, the reporters [14], [16]-[18] have been reported variations in the removal of these compounds, for example joss et al found the efficiency of removal of Ketoprofen 30% and Naproxen from 55 to 85%. This means that in addition to physiochemical proprieties of trace organics, their removal also depends on operations conditions such as the temperature [19], [20], the HRT [21] and the PH [22].

TABLE V
TRACE ORGANIC CONTAMINANT REMOVAL EFFICIENCY OF THE MBR OVER 275 DAYS OF OPERATION

Pharmaceutical compound	Influent (µg/L)	Removal (%)		References
		MBR	MBR-GAC	
Codeine	0,18	100	100	
Ketoprofen	6,4	97	100	(30%) [13]
Paracetamol	177	100	100	
Diclofenac	0,1	30	100	(5–45%) [14]
Naproxen	3,7	94,6	100	(55-85%) [14])
Ibuprofen	1,4	96,4	100	(90-100%) [14]
Roxithromycin	0,21	57,1	100	(<60%) [15]
Sulfametoxazole	1,6	18,8	100	
Metronidazole	4,8	56,3	100	15–18% [13]
Trimethoprim	1,4	14,3	100	90% [15]
Hydrochlorothiazide	2,8	10,7	100	
Furosemide	5,1	74,5	100	35–40% [13]
Caffeine	41	96,1	100	
Gemfibrozil	0	100	100	
Pravastatin	0,42	100	100	45% [13]
Metoprolol	0,1	10	100	
Atenolol	0,77	70,1	100	70% [13]
Acide Fenofibric	2,1	71,9	100	
Carboxyl-ibuprofen	16	100	100	80% [14]
Iopromide	1,1	74,5	100	60-80% [13]
Iohexol	167	99,8	100	

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Category	Compound	CAS number	Molecular weight (g/mol)	Log K_{OW} [a]	Log D at pH 7 [a]	Dissociation constant (pKa)[a]	Water solubility (mg/L)[b]	Charge	Structure of compounds
Pharmaceutically active compounds	Ibuprofen ($C_{13}H_{18}O_2$)	15687-27-1	206.28	3.50 ± 0.23	0.94	4.41 ± 0.10	21	Negative	
	Acetaminophen ($C_8H_9NO_2$)	103-90-2	151.16	0.48 ± 0.21	0.47	9.86 ± 0.13 / 1.72 ± 0.50	14000	Neutral	
	Naproxen ($C_{14}H_{14}O_3$)	22204-53-1	230.26	2.88 ± 0.24	0.73	4.84 ± 0.30	16	Negative	
	Ketoprofen ($C_{16}H_{14}O_3$)	22071-15-4	254.28	2.91 ± 0.33	0.19	4.23 ± 0.10	16	Negative	
Surfactants and industrial chemicals	4-tert-octylphenol ($C_{14}H_{22}O$)	140-66-9	206.32	5.18 ± 0.20	5.18	10.15 ± 0.15	5	Neutral	
	4-n-nonylphenol ($C_{15}H_{24}O$)	104-40-5	220.35	6.14 ± 0.19	6.14	10.15	6.35	Neutral	
	Bisphenol A ($C_{15}H_{16}O_2$)	80-05-7	228.29	3.64 ± 0.23	3.64	10.29 ± 0.10	120	Neutral	
Steroid hormones	Estrone ($C_{18}H_{22}O_2$)	53-16-7	270.37	3.62 ± 0.37	3.62	10.25 ± 0.40	677	Neutral	
	17-β-estradiol ($C_{18}H_{24}O_2$)	50-28-2	272.38	4.15 ± 0.26	4.15	10.27	3.9	Neutral	
	Diclofenac ($C_{14}H_{11}Cl_2NO_2$)	15307-86-5	296.15	4.55 ± 0.57	1.77	4.18 ± 0.10 / -2.26 ± 0.50	2.4	Negative	
	Primidone ($C_{12}H_{14}N_2O_2$)	125-33-7	218.25	0.83 ± 0.50	0.83	12.26 ± 0.40 / -1.07 ± 0.40	500	Negative	
	Carbamazepine ($C_{15}H_{12}N_2O$)	298-46-4	236.27	1.89 ± 0.59	1.89	13.94 ± 0.20 / -0.49 ± 0.20	18	Neutral	
	Salicylic acid ($C_7H_6O_3$)	69-72-7	138.12	2.01 ± 0.25	-1.13	3.01 ± 0.10	2240	Negative	
	Metronidazole ($C_6H_9N_3O_3$)	443-48-1	171.15	-0.14 ± 0.30	-0.14	14.44 ± 0.10 / 2.58 ± 0.34	9500	Neutral	

Fig. 3 Physiochemical properties of traces organics found in hospital waste water

C. Removal of Trace Organic Contaminants by MBR- GAC System

The removal effectiveness of the activated carbon adsorptive treatment system depends on the properties of the adsorbent (specific surface area, porosity, surface polarity, and physical shape of the material) and the characteristics of the compound (shape, size, charge and hydrophobicity). Adsorption mechanisms consist of the chemical (electrostatic interaction) and physical bindings of molecules to the surface of an adsorbent. The latter is often more important due to the capability to form multi-layer bindings [23]. In fact, it was recently reported that the greatest removal of amoxicillin by activated carbon was achieved under pH conditions corresponding to a zero net charge on the activated carbon surface [24].

The sorption efficiencies of organics traces to activated carbon may be significantly altered by several factors, such as the types of activated carbon used, the initial concentrations of target compounds and the pH, temperature and dissolved organic carbon (COD) concentration of the solution [25]-[28].

The capacity of activated carbon to adsorb a particular compound can, to some extent, be predicted based on the 'hydrophilic' or 'hydrophobic' nature of the chemical [23]. The hydrophobic (non-polar) or hydrophilic (polar) properties of pharmaceutics compounds can be determined from their LogD (or pKa-adjusted Log Kow) values. It has been reported that non-polar compounds with Log Kow > 2, may be effectively removed with activated carbon by hydrophobic interaction [23]. However, the adsorption of more polar or charged compounds to activated carbon is much more difficult to predict due to additional effects of polar interactions and ion exchange [23]. For that in our study we have been changed the ionic forces of activated carbon by treating with acidic and basic solution in high concentration. Many pharmaceutics compounds, such as tetracycline and sulfonamides are often present in negatively charged form at normal operating pH conditions [29]. Therefore, the use of ionic treatment processes may be effective for the removal of this anionic micropollutant [30] and that according with our results in this study. Ion exchange is the main mechanism in the ionic treatment for negatively and positively charged pharmaceutics.

Full-scale studies are required to determine the optimal configuration and operating conditions of adsorptive systems, which are effective and economically feasible for pharmaceutics compounds removal. In another side, in this study, we can confirm that initially GAC post-treatment could significantly improve the removal of the compounds which demonstrated low to moderate removal by MBR treatment (i.e., diclofenac, Roxithromycin, Sulfametazole, Hydrochloric-thiamine, Furosemide, Metoprolol, Atenolol, Acide Fenofibric, Iopromide, Trimethoprim, and metronidazole) see Table V.

IV. Conclusions

This study reported the stabilization of extern MBR system in biological treating of the hospital effluent during the operation a period over of 275 days. The results confirmed the high efficiency removal of COD and Nitrogen. The MBR system treatment can effectively remove Ketoprofen, Naproxen, Paracetamol, Ibuprofen, Caffeine, Gemfibrozil, Pravastatin, Carboxyl-ibuprofen, and Iohexol. The GAC column following the MBR treatment was demonstrated a high (95-100%) removal for all the organics traces in the hospital waste water. The ionic force of activated carbon and the electronic charge of organic micropollutant were two parts of chemical and electronic interaction which have been as important mechanism for complete and effective removal of organic micro pollutant of the waste water treated by MBR.

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Conclusion and Future Work

Conclusion and Future Work

➤ Conclusions

To study removal organic micropollutants in hospital wastewater by treatment systems as the MBR means to deal with a very complex set of problems. This thesis investigated in strategic research the logical steps to increase our scientific knowledge about many important details:

1. The effects of hospital wastewater on the treatment performance of the MBR and CAS systems.

2. Study the toxic impact of hospital effluents on the microorganisms and characterize the changes in composition the sludge to decrease the membrane fouling phenomenon under different reel operating conditions.

3. Development a MBR system to achieve high removal of organic micropollutants in treating the hospital effluents and produce a high quality effluent in the outlet.

This thesis began with literature review to demonstrate the significant of available data and for identity keys points for subsequent investigation. Few of studies was illustrated the potential effects of hospital effluents on the aquatic environment, microorganisms, the human health, and performances the wastewaters systems process and that explain reel difficult in this area. In addition to many of these studies was treated with synthetic wastewater and that means absence the reel conditions of treatment although presence the scientific according.

In this study, the reel conditions and the technical experiments with pilot- scales was the first gaol of our strategy to reach for high efficiency of removal organic micropollutants.

This thesis was succeeded to employed new technical instrument as confocal microscopic in morphologic characterisation of activated sludge. In addition to reach a qualitative and quantities analyses for the EPS and their composition by using special staining dyes. In

chapter 3 at article (1)" It can be concluded that CLSM, in combination with image analysis, is a powerful method for direct determination of the EPS distribution, heterogeneity factors and the structure of activated sludge flocs. This study also found that there is a good correlation between the chemical analyses of EPS and the statistical treatment of microscopic pictures". That was important to confirm our chemical analyses.

In anther side, in chapter 3 and article (2) the toxic effects of hospital effluents (HE) and their pharmaceuticals compounds by comparison with urban effluents (UE) on the bacteria (as microorganisms in direct contact with antibiotics residues), and performance the CAS system was a principal objective. We could conclude that:

1. HE leads to the erosion of sludge flocs, resulting in an increase in floc fragments, exposure of 'core filaments', and the degradation of the organic load of the treated effluent.

2. Denser floc matrices, observed by autofluorescence coupled with bacterial staining in the HE feed, suggested that during HE treatment EPS content of the flocs increased. It seems reasonable to infer that HE promoted EPS production by the biomass. This hypothesis is supported also by the observed increase in the proportion of important floc-forming bacteria in the HE reactor.

3. Structural divergence of the bacterial community in a lab scale CAS reactor and also a reduction of the bacterial diversity, while other reactor parameters, such as the nitrification rate, were not affected.

4. The introduction of Pseudomonas strains originated from HE in sludge could increase the risk of antibiotic resistance dissemination in WWTP processing HE. Furthermore, stress conditions, including antibiotics, also induce the acquisition of antibiotic resistance gene cassettes by RIs via the SOS response. HE offers ideal conditions to promote genetic evolution in bacterial communities.

From this important finding we have decided studding the MBR as a modern technology in treating the HE in based on the CAS system that mean a comparative study between the CAS and MBR systems for explain, exactly effect the HE in two process and performance both

systems in treating this type of effluents (In article 3). The analyses performed on the supernatant and activated sludge bioreactors allow us to draw the following conclusions:

- The MBR was able to achieve good organic removal efficiencies. MBR removal efficiencies based on T COD, S COD by comparison with the CAS.

- Despite the low concentration studied, the toxicity of the pharmaceutical compounds on activated sludge altered the characteristics of the biological matrix. The presence of the pharmaceutical compounds stimulated the mechanisms of survival (higher production of EPS). Fouling potential seems to be linked more closely to polysaccharides than other EPS.

- Simultaneously, confocal laser scanning observations and three-dimensional EEM spectroscopy showed significant modifications of sludge morphology. (Higher production of soluble EPS).

The results obtained of dosage the compounds pharmaceuticals showed that the MBR presented higher removal efficiencies than the CAS for almost of compounds.

This step of our research has been useful for give us the motivation a development and optimisation the MBR. That was in article (4) in" Application of membrane biofilm bioreactor (MBBR) for hospital wastewater treatment: Performances and Efficiency for Organic Micropollutant Elimination". This study reported that: the possible role of supports media in MBBR at decreasing production the proteins and polysaccharides in the soluble phase as result the fouling of membrane. This finding confirmed that the biofilm supports media could be played a major role in increasing the efficiency of MBR system in treating the hospital wastewater. But, that proposed strongly this question" What about the filtration by extern membrane and with full pilot- scale and for long time (different operation conditions)? This type of application could use in the medicals factories and hospitals in treating the HE before reach to WWTP. To answer scientifically we have studied" Upgrading the performances of Ultrafiltration Membrane system coupled with Activated Sludge Reactor by addition of biofilm supports for the treatment of hospital effluents" in article (5). This article was concluded that biofilm supports media addition in a biological system followed by ultrafiltration membrane had consequences on the global quality of the treatment with a slight increase of performance based on classical parameters, coupled to an important improvement of pharmaceuticals removal (pravastatin, ketoprofen, diclofenac, roxithromycin, gemfibrozil, codeine, Iohexol). This result was linked to the increase of

biomass concentration, of the solid resident time and of the sorption capacity. As it has been shown furthermore, the treatment of pharmaceuticals was due to the biological degradation depending at once of the biodegradability of each molecule (and with could be configured by a parameter Kbiol) and its sorption capacity (parameterized by the Koc), and, in our system by the porosity of the membrane. This membrane efficiency is function of the fouling phenomena, dependant of the quality of the influent and by washing operations. The occurrence of a biofilm system in a biological reactor has direct consequences on the quality of discharged effluent, retaining the suspended solid in the biological reactor and protecting the membrane. It was shown in this study, by biochemical analysis and confocal observations, that the development of biofilm in the system permits a modification of the proportion of the major exo-polymeric substances in the soluble phase compared to a free cells system. The concentration of proteins, identified as a cause of clogging in membrane system, decreases which induces a better stability of the transmembrane pressure. Consequentially, the decrease of operating cost could compensate the equipment cost.

But, in all our experiments we have discovered that it was impossible to have a complete removal without studding mechanism of removal for all organic micropollutants and their physical and chemical characterisations to find a new approach for high removal efficiency. In this area, there was many studies explained role of activated carbon in removal the organics micropollutants. From this title the research was to find common factor between all the organics micropollutant presented in the HE. That was the Ionitic force which motive the chemical function to take electric interaction in presence an adsorbent material has ionise special characteristics that means, by example, granular **modified** activated carbon. Article (6) studied "Efficiency of Modified Granular Activated Carbon Coupled with Membrane Bioreactor for Trace Organic Contaminants Removal". In this article The MBR system treatment can effectively remove Ketoprofen, Naproxen, Paracetamol, Ibuprofen, Caffeine, Gemfibrozil, Pravastatin, Carboxyl-ibuprofen, and Iohexol. The GAC column following the MBR treatment was demonstrated a high (95-100%) removal for all the organics traces in the hospital waste water. The ionic force of activated carbon and the electronic charge of organic micropollutant were two parts of chemical and electronic interaction which have been as important mechanism for complete and effective removal of organic micro pollutant of the waste water treated by MBR.

This thesis studied the effects of hospital wastewater on the treatment performance of the MBR and CAS systems. The toxic impact of hospital effluents on the microorganisms and characterize the changes in composition the sludge to decrease the membrane fouling phenomenon under different reel operating conditions.

The influence of biofilm supports media, modified activated carbon and separation technique (membrane) to achieve high removal of organic micropollutants in treating the hospital effluents.

Finally, I hope that this thesis shed new light on this important subject which will be direct risk on the public human health and it could find alive data for development and optimization of removal systems process.

➢ **Future work**

In this thesis I have been all the data bases and the operation conditions to have a mathematical model for optimisation the MBR in high removal efficiency of organics micropollutants. For that the recommendations studies for future work:

(1) Carbon adsorption: To complete my studding in development the MBR combined with powder activated carbon reactor and find a modelling constant adsorption in reel operation conditions.

(2) Magnetic separation : The combined use of magnetic field and iron-based complex in membrane bioreactor in treating the hospital wastewaters and in same area study the potential applications of the nanoparticules ,as the MFe_2O_4 magnetic, in removal the organic micropollutants.

(3) Biofilm: To study bio-mechanisms of microbial activity and bio-kinetics in the biofilm membrane bioreactor MBBR, and identifying the relation between the microbial

species and high removal efficiency of organic micropollutants in MBR system process.

Abstract

This research investigates the removal of pharmaceutics present in hospital wastewaters by conventional activated sludge and MBR systems of treatment and under various operating conditions to elucidate the removal mechanism and increasing the efficiency of removal. In this study, laboratory scales was composed to four types of reactors used: Bach reactors, conventional activated sludge, submerged membrane bioreactor and extern membrane bioreactor and all these reactors were feed in reel hospital wastewaters. Different Technical studies and many experiments were affected to develop the MBR systems: the beginning was with biofilm supports media and the attached growth of biofilms in the reactor and the finish by using the powder activated carbon. En general, the reported results show high performance for the MBR with compared to CAS system in treating the basic organic pollutants. Presence the biofilm supports media was very important for high removal of pharmaceuticals compounds from the hospital wastewaters. The presence of the pharmaceutical compounds stimulated the mechanisms of survival higher production of EPS. Fouling potential seems to be linked more closely to polysaccharides than other EPS. In this study, for the first time, was employed the confocal microscopy for qualities and quantities analyses for the EPS in the biologic reactors. Microscopic observations were confirmed the chemical analyses of EPS compounds.

In final experiment 21 pharmaceuticals were eliminated from the hospital effluents during the treatment in extern membrane (UF) with modified granular activated carbon. In addition to many biomolucles analyses which study the principals impact of hospital effluents on the microorganism's especially the bacteria in using different, recent techniques. This study demonstrates by reel conditions the role the developed MBR systems in treating the hospital effluents and its impact direct on the environment.

Keywords: Membrane Bioreactors, Biofilms supports, Hospital wastewater, EPS

Résumé

Cette recherche porte sur l'élimination des micropolluants pharmaceutiques des effluents hospitaliers par des procédés biologiques classiques (boue activée) et membranaire. Il est montré que les systèmes à membrane, externe ou immergée, permettent un meilleur traitement, ou une meilleure rétention, de plus de 50% des molécules pharmaceutiques mesurés. Afin d'améliorer l'efficacité des procédés membranaires, des supports bactériens ont été ajoutés dans le bassin biologique permettant de diminuer considérablement le colmatage. Il est montré qu'une des conséquences de la présence de ce garnissage est une diminution globale des EPS produits, donc du colmatage membranaire, et de la rétention des molécules pharmaceutiques,. Afin d'augmenter encore l'efficacité du procédé, du charbon actif en poudre ou en grain a été ajouté avant la filtration (CAP) ou en sortie de filtration (CAG), permettant une élimination quasi complète des molécules mesurées. La qualité des biomasses épuratrices a été suivie par microscopie confocale avec marquage fluorescent des exopolymères et de la viabilité cellulaire. Il est montré que les effluents hospitaliers modifient la structure des flocs et des biofilms, leur composition biochimique, avec une augmentation des concentrations en protéines extracellulaires, et la répartition des populations caractérisées par métagénomique.

Mots- clés : Bioréacteurs membranaires, biofilms supports media, effluents hospitaliers, EPS