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Etude comparative des matériaux de garnissage dans les réacteurs de filtration pour l'assainissement non collectif

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Abbreviation

AB:	Lewis acid-base interactions
aMW:	apparent molecule weight (kDa)
ATP:	adenosine triphosphate
ATR-IR:	attenuated total reflectance-infrared
AWI:	air-water interface
BOD_5:	biological oxygen demand at 5 days (mgO/L)
BSA:	bovine serum albumin
CER:	cation exchange resin
CFU:	colony forming unit
CLSM:	confocal laser scanning microscopy
COD:	chemical oxygen demand (mgO/L)
CSTR:	continuously stirred tank reactors
C_m:	missive water retention capacity
C_v:	volumic water retention capacity
D_{10}:	the screen diameter passing 10% of sand sample (mm)
D_{60}:	the screen diameter passing 60% of sand sample (mm)
DGGE:	denaturing-gradient gel electrophoresis
D_m:	average diameter of material grains (mm)
DNA:	deoxyribose nucleic acids
DO:	dissolved oxygen
EDL:	electrical double layer
EDTA:	ethylenediaminetetraacetic acid
EL:	electrostatic interactions
EPS:	extracellular polymeric substances
FDA:	fluorescein diacetate
FFF:	field flow fractionation
FISH:	fluorescence in situ hybridization
GC:	gas chromatography
GFP:	green fluorescence protein
HLR:	hydraulic loading rate
HPLC:	high performance liquid chromatography

(HP)SEC: (high pressure) size exclusion chromatography

HRT: hydraulic residence time

HS-like: humic-like substances

ICP-ES: inductively coupled plasma emission spectroscopy

K: permeability coefficient (mm/h)

k_s: saturated hydraulic conductivity (m/s)

LB-EPS: loosely bound EPS

LW: Lifshitz-van der Waals interactions

NMR: nuclear magnetic resonance

OM: organic matters

OWTS: onsite wastewater treatment system

PCR: polymerase chain reaction

PE: population equivalent

PFGE: pulse field gel electrophoresis

PN: proteins

PS: polysaccharides

RISA: ribosomal intragenic spacer analysis

RNA: ribose nucleic acids

RTD: residence time distribution

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: scanning electron microscopy

SPANC: service public d'assainissement non collectif

SRT: solids (sludge) retention time

STXM: scanning transmission X-ray microscopy

TB-EPS: tightly bound EPS

TKN: total kjeldahlnitrogen (mgN/L)

TOC: total organic carbon (mgC/L)

Tot N: total nitrogen (mgN/L)

t_s: infiltration time (s)

(T)SS: (total) suspended solids (mg/L)

UC: uniformity coefficient

VDW: volatile dry weight

(X)DLVO: (extended) Derjaguin-Landau-Verwey-Overbeek

3D-EEM : 3 dimension fluorescence excitation-emission matrix

Introduction

Contexte de l'étude

En France, l'assainissement autonome (OWTS) représente une voie d'assainissement lorsque les habitations ne sont pas en mesure d'être connectées au réseau collectif de traitement des eaux usées. Ce type d'assainissement représente environ 15 millions de personnes et 100 000 à 150 000 installations sont construites chaque année avec un nombre total qui ne cesse d'augmenter. Au-delà de la législation française sur la qualité de l'eau et des milieux aquatiques N ° 2006-1772 en vigueur depuis le 30 décembre 2006, les objectifs de traitement des eaux usées au niveau européen ont été fixées par la direction du Conseil Européen (directive N ° 91/271 / CEE). Cette loi fixe des exigences strictes sur la qualité des rejets d'eaux usées dans l'environnement ; les petites collectivités de plus de 2000 équivalents-habitants (PE) sont obligées d'organiser la collecte des eaux usées et de mettre en place un système de traitement. Dans les districts ruraux de moins de 2000PE, les collectivités sont également tenues d'organiser et de contrôler les systèmes autonomes de traitement des eaux usées avec la mise en place de SPANC (Service Publique d'Assainissement Non Collectif).

La configuration commune à tous les systèmes d'assainissement autonomes est la suivante : i) un système de collecte des eaux usées ii) une fosse toutes eaux pour le traitement primaire des eaux usées iii) un traitement secondaire par infiltration dans le sol ou par injection discontinue dans des systèmes de filtre à sable. Depuis mars 2007, les implémentations de ces dispositifs et d'autres installations de traitement sont décrites par XP DTU 64.1. Le choix et la conception des dispositifs autonomes sont souvent basés sur un savoir-faire et des règles empiriques. La connaissance scientifique et le développement technologique sont nécessaires pour améliorer la fiabilité et la durabilité de ces systèmes. L'absence de mise à jour des connaissances limite le développement des technologies de l'assainissement autonome et laisse de nombreuses questions rencontrées dans la pratique des SPANC sans réponse.

L'exploitation des sables alluviaux est aujourd'hui limitée dans un contexte réglementaire européen à travers des notions de continuité morphologique des rivières. Son utilisation dans certains domaines tend à être limitée, et des matériaux de substitution comme les granulats

concassés sont envisagés pour le garnissage de filtres. Cependant l'impact de l'application de ces nouveaux matériaux à la filtration d'effluents septiques est très peu documenté.

Éléments scientifiques de l'étude

Les systèmes d'assainissement autonomes

Les travaux de recherche sur les systèmes d'assainissement autonome ont débuté au milieu du 20e siècle et ont concerné essentiellement les mécanismes de rétention et de dégradation dans les sols ou dans les lits filtrants constitués de matériaux granulaires et surtout de sables. Ils ont également permis de déterminer les conditions opératoires et d'exploitation des filtres afin de prolonger leur durée de vie et notamment dans le cas du traitement secondaire par infiltration-percolation dans des parcelles de sol ou sur les lits de filtration. Les fosses toutes eaux sont en mesure de réduire très largement les matières en suspension par décantation, réduire la quantité d'azote par digestion anaérobie et ammonification. Cependant, les effluents septiques sont toujours considérés comme l'une des raisons la plus fréquente de l'eutrophisation des milieux aquatiques et de la contamination eaux souterraines (Withers *et al.*, 2011; Stanford *et al.*, 2010). L'existence d'un traitement secondaire par infiltration dans le sol ou sur des tertres garnis de sable permet, avec des conditions d'écoulement insaturés, l'amélioration de l'épuration de l'effluent en engageant différents mécanismes dans le milieu poreux: filtration mécanique des matières en suspension, dégradation biologique de la matière organique par la biomasse hétérotrophe développée au sein du matériau filtrant, conversion de l'azote organique ou l'ammoniac en ions nitrates par la biomasse autotrophe également présente dans le réacteur de filtration (Van Cuyk *et al.*, 2001;. Rodgers *et al.*, 2005; Gill *et al.*, 2009). Les lits de filtration en écoulement insaturé permettent également la réduction des microorganismes par rétention mécanique, adsorption aux interfaces solide/eau ou gaz/eau ou par prédation par des protozoaires (Wan *et al.*, 1994; Stevik *et al.*, 2004; Chabaud *et al*, 2006). Les tertres d'infiltration, notamment les filtres à sable sont les plus recommandés et couramment installés en raison de leurs bonnes performances, leur facilité d'installation, et pour les aspects économique et esthétique.

Même si les mécanismes de dégradation de la matière carbonée de l'élimination de l'azote et du phosphore ont fait l'objet d'études, les informations concernant la nature de la biomasse et du biofilm développé sur les supports utilisés en assainissement autonome sont incomplètes. Les principales approches sur le biofilm ont été motivées par un élément de

dysfonctionnement des filtres fréquemment rapporté, le colmatage. Les travaux menés dans les années 2000 ont concerné le développement de la biomasse totale et parfois des éléments qualitatifs du biofilm : protéines, polysaccharides. Ces travaux ont montré des modifications du biofilm en fonction de la température, du temps de fonctionnement et suivant la profondeur du réacteur filtrant (Le Bihan & Lessard, 2000; Ragusa *et al.*, 2004; Zhao *et al.*, 2009). La matrice des EPS contient diverses macromolécules biologiques (protéines, polysaccharides, les lipoprotéines, glycoprotéines ... synthétisées par la biomasse cellulaire ou issue de la lyse des cellules) ainsi que les composés organiques adsorbés de l'environnement (substances humiques-like, substrats, minéraux) (Frølund *et al.*,1995; Higgins & Novak, 1997). Des auteurs ont affirmé que la présence d'EPS induit le colmatage du filtre ou une conductivité hydraulique réduite (Vandevivere & Baveye, 1992). Cependant, la compréhension du rôle et de l'implication négative ou positive des matériaux dans le processus de développement du biofilm et dans la production des EPS reste très limitée.

Facteurs modifiant le biofilm

Le rôle des matériaux filtrants sur le développement d'un biofilm ou sur la structuration de celui-ci dont les constituants de la matrice extracellulaire, a également été documenté (Rodgers *et al.*, 2004a; Kim *et al.*, 2010). Ces travaux montrent une influence de la taille des matériaux sur la quantité totale de biofilm présente et une modification par la vitesse du fluide de la structure du biofilm.

Le temps de rétention des solides (SRT) ou l'âge des boues, a également un effet considérable sur la production d'EPS, mais les résultats rapportés dans la littérature sont quelque peu contradictoires. Des chercheurs ont constaté que la quantité d'EPS dans divers agrégats microbiens augmente avec une augmentation du SRT, ce qui implique que les bactéries produisent plus d'EPS dans des conditions endogènes. Sesay *et al*. (2006) ont constaté que l'augmentation du SRT était positivement corrélée avec la quantité totale d'EPS dans des boues activées ainsi qu'avec la quantité de protéines et de glucides contenue dans les EPS. Le rapport entre les protéines et les sucres augmente également de 1,5 à 2,5 avec une augmentation de la SRT de 4 à 20 jours. Cependant, certains chercheurs ont suggéré que la production d'EPS est indépendante du SRT. Liao *et al*. (2001) ont constaté que la teneur totale en EPS n'a pas changée significativement avec une augmentation du SRT alors que le ratio protéine/glucide augmente lorsque le SRT est porté de 4 à 12 jours et reste inchangé alors que le SRT a été augmentée de 12 à 16 jours. Li & Yang (2007) ont rapporté que les

TB-EPS (tight bound EPS) de boues activées n'ont aucun rapport avec le SRT, mais que la quantité de LB-EPS (loosely bound EPS) diminue avec une augmentation de SRT.

L'hydrodynamique du réacteur a également été considérée via le régime d'écoulement (cisaillement). La force des cisaillements dans un réacteur peut également influencer la composition en EPS. L'augmentation du taux de cisaillement (dF/dS) ou l'augmentation de l'intensité de l'aération dans un réacteur SBR (réacteur batch séquentiel) semble augmenter la quantité d'EPS par unité de masse de boues (Adav *et al.*, 2007). Des travaux réalisés sur ce même type de réacteur, montrent que la quantité de polysaccharides extraite des boues activées augmente avec le taux d'aération alors que le celui de protéines reste constant (Shin *et al.*, 2001). Ces résultats montrent donc un comportement différent des bactéries soumis au cisaillement. Ramasamy & Zhang (2005) ont montré qu'une augmentation brutale des forces de cisaillement entraine une augmentation de la quantité de sucres contenue dans les EPS, mais après un certain temps la composition des EPS retrouve son état initial. La libération des EPS des boues dans le milieu peut être accentuée dans certaines conditions hydrodynamiques par un effet mécanique de cisaillement (Aquino & Stuckey, 2004; Sheng *et al*., 2006a).

Les conditions d'aérobie ou d'anaérobie peuvent également modifier la production d'EPS avec l'observation d'une diminution du taux d'EPS dans des boues activées dans des conditions d'anaérobie (Nielsen *et al.*, 1996). La désintégration de boues activées dans des conditions de carence ou de limitation en oxygène a été observée (Wilén & Balmér, 1998, 1999 ; Gaval & Pernelle, 2003). Ce phénomène pourrait être attribué à l'arrêt de la production d'EPS ou à l'hydrolyse de celles-ci. Shin *et al*. (2001) a comparé la production des EPS par des boues activées dans trois bioréacteurs et ont observent qu'un taux d'oxygénation augmente la production de polysaccharides avec le temps alors que le taux de protéines reste inchangé. Pour de faibles taux d'oxygénation, les concentrations de polysaccharides comme de protéines sont inchangées.

Problématique et objectifs de l'étude

Comme nous venons de le voir, l'hydrodynamique, l'oxygénation ou l'âge des boues sont des paramètres de la quantité et de la qualité du biofilm dont les EPS. Ces éléments sont susceptibles de modifier deux critères essentiels de l'évaluation d'un système d'assainissement autonome, i.e. les rendements épuratoires et le colmatage du filtre. La nature du garnissage est susceptible d'impacter l'hydrodynamique d'un réacteur qui, par son

fonctionnement insaturé, modifiera les conditions d'oxygénation du milieu et par un cisaillement plus ou moins important du biofilm, probablement l'âge des boues. La substitution du sable de rivière par des granulats peut donc entrainer des modifications majeures dans le fonctionnement et donc dans l'efficacité des massifs filtrants. Peu d'études ont été réalisées avec des matériaux concassés issus de carrières et aucune n'a fait l'objet d'une approche du fonctionnement biologique d'un filtre comparativement au sable. Liénard *et al*. (2001) a comparé les caractéristiques de matériaux de substitution et de sable comme la taille des grains, la porosité et la présence de particules fines. Une étude de l'échelle du laboratoire à court terme a été axée sur l'efficacité du traitement et de la biomasse sur agrégat écrasé (Wanko *et al.*, 2005).

Ainsi, même si le rôle de la structure des matériaux sur le comportement de la biomasse fixée est méconnu, la modification des matériaux va modifier des éléments de l'hydrodynamique et donc les conditions de développement du biofilm et des EPS liées à ce biofilm.

Afin de mettre en évidence un comportement différent des filtres en fonction de la nature du garnissage, nous avons donc choisi de concentrer nos expériences et nos observations sur des paramètres globaux de fonctionnement (rendements épuratoires) et sur des éléments apriori plus sensibles comme le biofilm et son organisation. Ces derniers éléments seront interprétés en tenant compte des caractéristiques des matériaux et de l'hydrodynamique des réacteurs.

Méthodologie et organisation de la thèse

La méthodologie retenue pour ce travail est une approche comparative d'installations pilotes sur une longue période afin d'observer des différences de comportement dans un régime pouvant être interprété comme stationnaire. Cette expérimentation longue permet d'observer d'éventuelles différences de comportement de réacteurs soumis aux mêmes conditions extérieures (T°, nature de l'alimentation) et donc à leur même variabilité. Cette expérimentation longue, sera validée par des duplicatas de colonnes garnies par les mêmes matériaux mais de hauteurs différentes, la hauteur ne devant pas interférer sur la colonisation du réacteur (pour une profondeur identique dans le lit). Des effets de charges volumiques (expérimentation à deux charges volumiques) doit permettre d'accentuer l'expression de certaines différences dans le comportement des matériaux (accumulation/rétention/croissance de biofilm).

Quatre matériaux ont été choisis : deux sables et deux granulats concassés avec 1 sable et 1 concassé dont les diamètres moyens sont proches.

L'approche méthodologique retenue est donc :

- Etude comparative des caractéristiques des matériaux entre les sables des rivières et des granulats concassés et impact sur l'hydrodynamique;
- Comparaison des capacités et de l'efficacité épuratoire des 4 matériaux choisis.
- Comparaison de la production et de la qualité du biofilm et des EPS et facteurs d'influence apportés par les différents matériaux.

Afin de répondre aux objectifs, ce travail de thèse est présenté en quatre parties :

- La première partie présente un état de l'art sur les réacteurs filtrants dans le cadre de l'assainissement non collectif. Les données concernant ce procédé et les caractéristiques des matériaux de garnissage sont discutées dans cette partie. Les mécanismes et les principaux facteurs contrôlant l'épuration de l'effluent septique dans un système de filtration sont évoqués en détails. Les mécanismes du colmatage biologique, le développement du biofilm, la matrice extracellulaires et ses composants structurel majeurs sont ensuite présentés. La fin de cette partie est consacrée aux moyens de caractérisation du biofilm (populations et composants structurels) afin de nous orienter sur des choix méthodologiques.

- La seconde partie est consacrée à la caractérisation des matériaux filtrants et à leur mise en œuvre en réacteurs. Le comportement des différents réacteurs matériaux a également été intégré à cette partie. Quatre matériaux, deux sables roulés et deux agrégats concassés, sont caractérisés et comparés en fonction de paramètres de forme et de compositions minérales. Les réacteurs sont étudiés notamment sur leur comportement hydrodynamique qui est représenté par le temps de séjour en écoulement discontinu.

- La partie suivante est consacrée à l'étude comparative des efficacités épuratoires des différents matériaux et pour différentes conditions opératoires. Cette partie est présentée sous forme d'un article :

- *Evolution of purification efficiencies of different filter materials and impact of filtration packing height and hydraulic loading (Soumis le 6 Novembre 2015 à Journal of the Taiwan Institute of Chemical Engineers : JTICE-D-15-0153)*

- La dernière partie est consacrée à l'observation et à la discussion des effets des matériaux sur le développement du biofilm avec une approche sur la biomasse totale et sur les composants majeurs de la matrice extracellulaire en fonction du temps de fonctionnement. La composition du biofilm dans la profondeur des réacteurs est également abordée. Cette partie est présentée à travers 2 articles :

- *Evolution of biochemical compositions of biomass developed in different aggregates for the use of filter materials of onsite wastewater treatment systems, (Soumis le 25 Avril 2015 à Biochemical Engineering Journal: BEJ-D-15-00418)*
- *Production of the evolution of extracellular polymeric substances (EPS) from the biofilm in onsite wastewater filtration reactors (Sera soumis à Bioresource Technology)*

Une conclusion générale développée et rédigée en français reprend les principaux résultats et propose une discussion autour des observations et des hypothèses importantes liées aux études expérimentales. Les perspectives des futures études sur le réacteur filtrant sont également évoquées.

Part I: Literature review

Preamble:

In this chapter, a literature review offers an overview of relevant and important studies in the research area of on-site wastewater treatment systems. It revisits the current knowledge on the treatment plants of domestic sewage in decentralized areas, including backgrounds, technique choices and treatment efficiencies. It also reviews the challenges encountered in the practice of vertical drained sand filters such as the clogging problem. It allows summarizing the various mechanisms involved in the process under unsaturated conditions of sand filter, and finally brings out the objectives and focus of this study. This chapter also includes currents studies on biological aspects of biofilm and its compositions and properties resulted from wastewater purification process.

I.1. On-site wastewater treatment systems (OWTS)

OWTS concerns 12 to 15 million people in France. According to French Standard of 07 September 2009, OWTS are defined as the technologies insuring the collection, the transport, the treatment and the evacuation of domestic wastewater from the buildings that cannot be connected to municipal wastewater network. This order also gives the prescriptions of applicable techniques for on-site treatment plants receiving a raw organic pollutant charge inferior or equal to 1.2 kg/j of BOD_5 (Arreté 07/09/2009, mortified by Arreté 07/03/2012). The technique choices and solutions are summarized by French standard NF DTU 64.1 (AFNOR, 2011). The OWTS are generally categorized as soil (or reconstructed media) infiltration systems and other installations with other treatment dispositive (micro-station, rotating biological disk...). The qualities of treated effluent request to be in accordance to receipting environment requirements: BOD_5 <35mgO/L and TSS <30mg/L.

I.1.1. Domestic wastewater (Raw water)

The domestic wastewater is produced by inhabitation. The flow rate of effluent collected from the outlet of an individual house in France varies from 80 to 180 L/habitation/day. The domestic wastewater is the combination of 2 major waste sources: about 25% of the effluent is toilet waste (blackwater) from WC flushes concentrated in human excreta such as faeces,

urine and companied by toilet paper disposal; the rest (about 75%) combines wastewater (greywater) from other sources: bath, shower, wash basin, kitchen sink and washing machine (Almeidaet *al.*, 1999). Blackwater and greywater have different characteristics, but both contain pollutants and disease-causing agents that require treatments. The black water contributes the majority of organic pollutants and pathogenic agents. The greywater brings in about 70% of the total volume that contains detergents, washing powder, and greases (Chocat, 1997). Eriksson *et al,* (2002) indicated that there were about 900 different xenobiotic organic compounds in greywater.

The domestic wastewater has similar compositions to municipal wastewater. The wastewater components that most wastewater facilities are designed to remove, are suspended solids, organic matters, nutrients and pathogenic organisms:

- Suspended solids (SS) consist of inorganic and organic materials. The suspended solids must be significantly reduced by treatment or they can increase the chemical oxygen demand (COD) when discharged to receiving water and provide sorption sites for microorganisms and other pollutants. They can also clog the soil adsorption fields in onsite systems.

- Organic matters (OM) in wastewater are traditionally characterized by COD, total organic carbon (TOC) and biological oxygen demands (BOD) and are often composed of particulate and dissolved fractions (size). However, the chemical compositions of OM are highly heterogeneous and are ranged from simple compounds like acetic acids to more complex polymers. The major chemical fractions in organics of wastewater are proteins, lipids and sugars, and their fractions can be changeable according to various conditions (Raunkjær et *al.,* 1994). Huang et *al,* (2010) indicated that the largest organic group in domestic wastewater was fibers (major components characterized as cellulose, hemicelluloses and lignin) which represented 20.64% of TOC, followed by proteins (12.38% of TOC) and sugars (10.65% of TOC), and the sum of volatile fatty acids, soluble proteins and soluble sugars represented about 30% of total COD.

- Typical nutrients in domestic wastewater comprise of inorganic and organic compounds of nitrogen and phosphorus, and also salts of certain metals (Na, Ca, K, Mg, Co, Mo, Cu, Mn). Nitrogen is reported as the sum of organic nitrogen, nitrate salts and ammonium. In a household, over 85% of the nitrogen load originates from the blackwater, ranging from 130 to 180mg/L (Palmquist & Hanaeus, 2005). Concentrations of Total Kjeldahl Nitrogen (TKN) in greywater range from 1 to 50mg/L (Eriksson *et al.,* 2002). The phosphorus presents in forms as orthophosphates, polyphosphate, and organic phosphorus (Crites & Tchobanoglous, 1998). The human body wastes, especially the urine are the major source of nitrogen and phosphorus. Food residue, laundry detergents, personal care products and cleaning products also contribute to the concentration of nitrogen and phosphorus and the presence of certain metals.

- The main source of microorganisms in domestic wastewater is human feces. Various types of microorganisms present in the domestic wastewater: bacteria, virus, and protozoa (John & Rose, 2005), including:

  - Bacteria: *Escherichia coli,* (*E. coli*), *Salmonella spp*., *Shigella spp.*, *Vibrio cholera*, *Yersinia*, *Pseudomonas* (Matthess & Pekdeger, 1985);
  - Virus: *Poliovirus*, *Rotavirus*, *Hepatitis*, *Echovirus*…(John & Rose, 2005);
  - Protozoa: *Giardia lamblia*, *Entameobahistolytica*, *Cryptosporidium parvum* (Macler & Merkle, 2000).

The main contaminants contents vary with the countries and their own living habits. The data of the concentrations of the critical characteristics of domestic wastewater must be summarized from three studies in France (Table 1.1):

Table 1.1 : Data (average values) of critical contaminants in domestic wastewater

Historic data	(Gougoussis, 1982)	(Maillard, 1998)	(Lakel, 2009)
Physical characteristics			
pH [-]	-	6.6	-
TSS [mg/L]	367	343	337
Organic characteristics			
BOD_5 [mgO/L]	621	327	289
COD [mgO/L]	1300	930	670
Nitrogen and phosphorus characteristics			
$N\text{-}NO_3$ [mgN/L]	0.74	0.33	-
$N\text{-}NH_4$ [mgN/L]	72	74	-
Total N [mgN/L]	-	91	68
$P\text{-}PO_4$ [mgP/L]	Range: 13-26 mg-P/L according to Lowe *et al.*, (2007)		
Microbiological characteristics [CFU/100mL]			
Total coliform	-	-	1.9×10^7
Thermotolerant coliform	3×10^7-1×10^{10}	1.1×10^7	-
E. coli	-	-	8.7×10^6
Enterococci	4×10^5-5×10^8	5.5×10^6	4.3×10^6

I.1.2. Primary treatment and Septic effluent

❖ *The fate of main pollutants in septic tank*

The raw wastewater is generally collected, transported and pre-treated by septic tank. A septic tank is a buried watertight container that served as the combination of settling tank and unheated unmixed anaerobic digester. The pre-treated effluent is called septic effluent. The septic tank insures the separation of floated materials and most of settable solids from liquid, and certain solids and non-settable organics undergo the decomposition by anaerobic digestion. The anaerobic digestion can be separated into two steps: fermentation (results in organic acids) and methanization (results in biogas). During the fermentation, the hydrolysis of complex organic matters firstly takes place and leads to an incomplete degradation of these organics into simple monomers; secondly these monomers are degraded directly into acetones or biogas (CO_2, H_2); or undergo the acidogenesis and results in organic acids (propionic acids,

butyric acids, acetic acids); During the methanization, the organic acids or monomers undergo an acetogenesis and a methanogenesis, then finally degraded into biogas (CO_2, CH_4). However, in septic tanks, methanogenesis bacteria hardly survive, and fermentation process predominates. This fact results in an important accumulation of partly degraded organics and volatile grass acids (Philip, 1983).

Before entered in septic tank, 73% of nitrogen presented in domestic water is organic nitrogen and 24% exists as ammonium. The nitrogen in raw wastewater is partially treated in conventional septic tanks due to the anaerobic conditions. During this process, much of organic nitrogen is converted to ammonium known as ammonification; as a result, the nitrogen in septic effluent is 70-90% of ammonium and 10-30% of organic nitrogen (Lowe, 2007). Some removal (20-30%) of phosphorus in raw wastewater is attributed by the accumulation in residual sludge that settled at the bottom. As a result, the septic effluent still contains 80-100% of phosphorus concentration found in the raw wastewater (Lowe & Siegrist, 2008). The removal of microorganisms is also relatively low. Table 1.2 summarizes the literature data of the main physico-chemical characteristics and indicator bacteria levels in septic effluent:

Table 1.2 : Main characteristics of septic effluent

Authors	Van Cuyk *et al.* 2001	PhD thesis: Chabaud. 2007	Lakel. 2009	Kauppinen *et al.*, 2014	Siegrist *et al.*2014
Average values: Suspended solids [mg/L] and organic characteristics [mgO/L]					
TSS	69	99	74	37	26
BOD_5	227	231	-	-	158
COD	386	290	343	320	226
Nitrogen and phosphorus characteristics [mgN/L]					
$N\text{-}NH_4$	47	71	48	-	52
Total N	57	66	-	72	51
Total P	4.6	3	-	9.2	23
Microbiological characteristics [CFU/100mL]					
Total coliform	-	6.4×10^4	-	-	-
Thermotolerant coliform	5.4×10^5	-	-	-	3.9×10^5

❖ *Environmental impact of septic effluent*

The widespread use of septic tank in suburban and rural areas contributes the disposal of septic effluent in receiving environments, and becomes one of concerns of groundwater, surface water and soil contaminations that are susceptible provoking waterborne diseases. In United States, septic tanks are one of the most frequently reported sources of groundwater contaminations (Yates, 1985). The septic tank effluents which contain phosphorus and ammonia are also potential source of eutrophication risk in rural aquatic environments, and the ammonium and the phosphorus absorbed by the sediments may become a source of nutrients for submerged macrophytes (Withers *et al.*, 2011). The organic matters partially removed by anaerobic digestion remain in septic tank and septic effluent. The steroid estrogens and nonylphenols remained in septic effluent have the possibility of penetrating in soil adsorption systems and being transported into groundwater (Stanford *et al.*, 2010). Septic effluent or pre-treated domestic wastewater, being highly risky to natural environment contaminations, should not be discharged directly and further treatments are necessary. The secondary treatment of domestic wastewater should respond to the conceptions of on-site sanitation: the treatment should be built in situ and technically simple to be carried out, controlled and monitored. The treatment should not also consume much energy. The design should not affect the landscape, etc.

I.1.3. Secondary treatment of OWTS – Soil infiltration systems

The secondary treatments of OWTS or the treatments of septic effluent are generally based on soil adsorption fields by using the infiltration and purification capacities of the natural soil. The field is sometimes reconstructed by other packing materials having similar structure but with characteristics more suitable for the treatment.

The natural soil adsorption are generally referred to soil infiltration trenches (underground), spreading bed of small depth (underground) and infiltration mounds (aboveground). The principle of these different configurations is similar and it is based on the infiltration – percolation theory: the effluent percolates through several meters in the unsaturated zone and at the same time combining the purification phenomena. The purified effluent will be recharged in the water table, or will be drained outside.

The structure and the composition of soil decide the soil permeability. Natural soil is a mixture of various kinds of grains: clay, silt, sand, gravel, etc. The soil texture impacts physically and chemically the functioning of infiltration system: a clayish soil indicates a better chemical capacity of pollutants, but worse physical behaviors (poor porosity, low permeability, terrible air exchange ability…); a sandy soil presents a higher permeability and a better air exchange, but a weak adsorption capacity (Hillel, 1988). The permeability of soil *K* is determined by the following equation:

$$K = \frac{\text{Quantityofwaterinjected } (m^3)}{\text{Infiltrationsurface } (m^2) \times \text{Testperiode } (h)} \ (mm/h)$$

The permeability *K* varies with the texture of the soil, thus the permeability. The following table (Table1.3) summarizes the values of *K* and the permeability for some textures of soils:

Table 1.3 : Values of permeability coefficient K of different textures of soil (NF DTU 64.1)

Texture of soil	Permeability coefficient *K* (mm/h)	Permeability
Clayish soil	<6	Impermeable
Clay-silty soil	6-15	Weakly permeable
Silty soil	15-30	Poorly permeable
Sandy-silty soil	30-50	Correctly permeable
Sandy soil	>50	Strongly permeable

According to NF DTU 64.1, for a clayish soil with $K<10$, the infiltration-percolation systems by natural soil are not recommended. In such case, infiltration systems can be designed with other packing materials, such as constructed wetlands, horizontal or vertical drained sand filters with or without plants. Among these systems, single pass vertical drained sand filter with discontinuous dosing is preferred, due to its good performances against minor energy consumption.

I.1.4. Current problems of vertical unsaturated sand filter

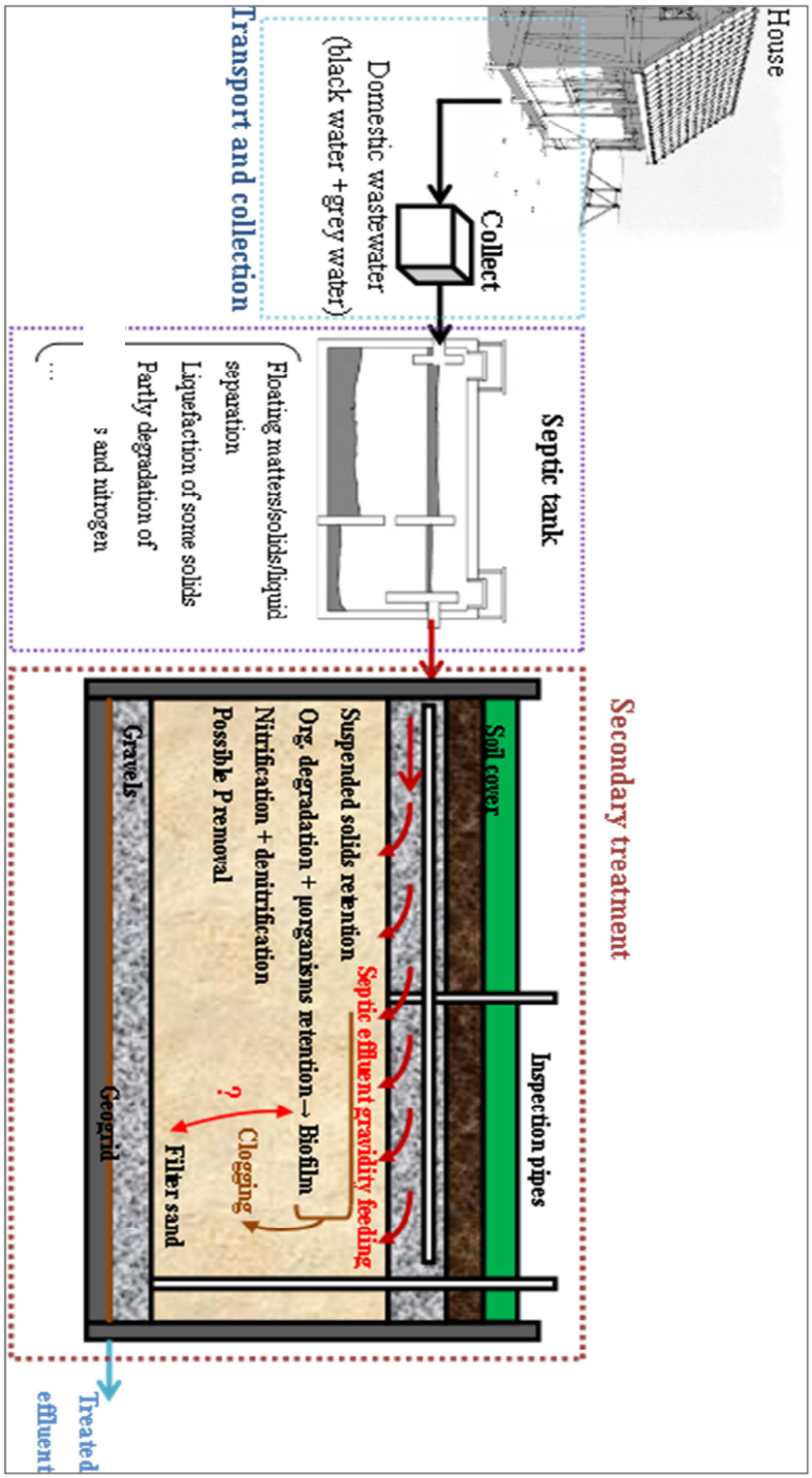
❖ Filter sizing is empiric. The lack of scientific base of filter conception often leads to oversized filtration beds in order to meet the potential needs of long-term functioning. Efforts and criteria have been made with the purpose of finding the correlation between filter sizing and long term functioning (clogging) since 1970s: Authors tented to propose the operation conditions and sizing criteria that provide a balance between necessary nutrients loads and

clogging provoked by organic and particulate matters accumulation (Healy & Laak, 1974); Later, design criteria have been improved since more full scale soil infiltration beds and sand filters have been investigated (Jenssen & Siegrist, 1990).

❖ Packing materials: not all the granular materials can be used in sand filter. Proper, durable, inert and granulometric well defined materials are required. In general, the alluvial sands are recommended. However, due to the excessive exploration, the alluvial sands are less available that drives more attention to other suitable, potential materials, for example, the crushed aggregates resulted from massive rocks reducing is nowadays considered as appropriate substantial choice, however their characteristics still need analysis and comparisons to the common materials. The characteristics of materials are discussed in the next chapter.

❖ Clogging: filtration system failures reported are mainly caused by clogging. Investigations of clogging lead to three major mechanisms: pore blocking caused by suspended solids (DeVries, 1972); over developed biomass and accumulation of extracellular polymers substances, such as proteins and polysaccharides (Pell & Nyberg, 1989b); and precipitation of certain metals such as FeS and $CaCO_3$ (Cooke *et al.*, 1999). Fewer studies have based on the clogging process and packing materials characteristics. How the materials of different nature impact the development of clogging or the purification efficiency remains unclear. The mechanisms of clogging will be discussed in details in following chapters. A global view of OWTS systems based on infiltration fields is shown in Figure 1.1:

Figure 1.1: Global view of OWTS system based on sand filtration fields



I.2. Characteristics of packing materials: river sands and crushed aggregates

Granular materials can be classified into several groups according to their origins, production processes and physical properties.

According to their origins, these granular materials can be separated into three categories: alluvial granular (river, marine sand and gravel); granular of massive rock (granites, diorites, basalts, and limestone); artificial and recycled granular (recycled concrete, construction or industrial by products).

Different origins of sands and production techniques result in different physical, chemical properties and hydraulic behavior of sand. The impacts of different sands on the filtration mechanisms and clogging development are not well known.

I.2.1. Origins and production of fine granulates

1) Origins

The most common deposit of alluvial sands is river beds or old river beds. The river sands and gravels are deposited by water flow, based on siliceous or siliceous-calcareous chemical composition; marine alluvial sands are mainly represented by deposits of ancient fluvial coastal beds. These gravels are immerged about thousands years since the last sea level rise; besides the hydraulic force provided by rivers or seas, glacial alluviums are accumulated during glacial periods of Quaternary.

Crushed gravels are manufactured from a series of exploitations and treatments of massive rocks and the origins of these rocks can be divers: sandy layers, limestone, sandstone exploited from sedimentary basins or maintains; hard metamorphic rocks (ex: quartzite, gneiss) from the ancient maintains; eruptive rocks (ex: granite, diorites) developed from crystal plutonic or volcanic.

2) Production of granulates

Five major steps intervene in the process of sand production: (1) cleaning the non-exploitable layers; (2) extraction of materials; (3) transport to the treatment site; (4) treatments of granulates until getting the fine products; (5) restoration of exploited site. The

main difference of production process between alluvial sands and crushed sands remains on the extractions and treatments.

First of all, the extraction of alluvial sands can be effectuated directly, however, for compact deposits like massive rock, the explosive have to be employed. One explosion can result in cutting down of a large quantity of rocks (more than 10 000 tons). Then exploded rocks are transported to the treatment site for following operations: crushing, cleaning, and screening in order to get the desired granulometry.

The crushing is performed by crushers in order to reduce successively the various sizes of raw materials. There are several types of crushers: jaw crushers, gyratory crushers, cone crushers, impact crushers (horizontal shaft impactor and vertical shaft impactor). The principles of these crushers are similar: the force is given mechanically by rock breakers (jawstock, eccentrically gyrating spindle, or hammers), in order to break the rocks into smaller pieces before going into the screening machines. Screening (or sieving) allows selecting the grains. The sieve permits only to let pass the elements inferior of certain size. Granulates of desirable size can be chosen and obtained by several time of crushing and screening. The washing has to be carried out in order to remove dusts during production process and keep the final products clean which is necessary to industrial utilizations. The operations of screening and washing are often combined together under water ramps during the sieving. Once granulates are reduced, treated, and classified, the products are transported to storage areas.

I.2.2. Physical characteristics of materials

- *Granulometry and effective diameter*

Granulometric analysis (EN 933-1, AFNOR, 1997) permits the determination of material grain sizes and the distribution of grains in a granular material. This analysis consists of fractioning the material by screens with different opening which are defined by European standard EN 933-2. With granulometric analysis, some parameters can be determined, such as effective diameter, average diameter, uniformity coefficient, and fines particles contents. The effective diameter is defined as the screen opening letting pass 10% of sand sample which is referred to D_{10}, similarly D_{50} and D_{60} are also defined. For the infiltration, most studies have agreed that the d10 should be at least 0.2mm (Gold *et al.*, 1992; Guilloteau, 1994). The ideal d10 is recommended in the range of 0.2–2mm by DTU 64.1.

❖ *Uniformity and porosity*

Both the river sand and crushed aggregates presents the variety of grain size, with less or more fine particles filling in the space between larger grains. The Uniformity coefficient (UC) is defined as the ratio between D_{60} (the screen diameter passing 60% of sand sample) and D_{10}, estimated from the grain size distribution. The sand is less uniform with larger UC. The recommended UC value is between 3 and 6 in France. Sands with higher UC value are more heterogeneous and more unpredictable. They may contain tortuous path for water to move through, thus different hydraulic and hydrodynamic behaviors may be observed. A good filter media should have large porosity to amplify air exchange (Ball, 1994). Studies have showed that the degree of heterogeneity has less impact on infiltration rate than D_{10} and fines content (Liénard *et al.*, 2001). The UC also has less impact on the treatment performance than the grain sizes (Darby *et al.*, 1996).

❖ *Fine particles content*

The recommended fine particles content does not exist. But the fine particles content in sand could interfere the infiltration process: Revil & Cathles(1999) indicated that the permeability of a clayish sand decreases with the increasing of fine clay particle percentage. Then authors revealed by studying different types of sands, that the infiltration rate decrease in the sands with higher fine particles content thus the permeability also decreases (Liénard *et al.*, 2001). This study recommended also the utilization of sand with fines content inferior of 2.5%.

❖ *Particle shape*

The shapes of material grains are determined by mechanical and chemical forces. Chemical action and abrasion increase with age and older sands tend to be rounder regardless of particle size. The larger the particle (typically in grain size>0.4mm) is, the higher imperfections and brittle fracturing. Conversely, smaller particles are stronger because of the lack of imperfections (Margolis & Krinsley, 1974).

I.2.3. Impact of physical characteristics

According to French standard DTU 64.1, the grain size distribution is the key to sand filter design and functioning. The grain size manages the percolation behaviors and

purification behaviors: the larger the grains result rapid percolation, however, hydraulic residence time and pollutant treatment efficiency reduce (Boller & Kavanaugh, 1995). In the other hand, a filter of finer material is more fragile to the clogging: a contrast study showed that compared to coarse sand (D_{10}=0.8mm), the oxygen content decreased over time in finer sand (D_{10}=0.35mm) which led to the unequilibrium between oxygen diffusion and consumption. On contrary, for the coarse sand, oxygen content is always stable even during continuous loading (Rolland *et al.,* 2009). Authors proposed a narrower range of D_{10} ($0.2<D_{10}<0.4$) in order to insure an effective treatment (Liénard *et al.,* 2001).

I.2.4. Comparison between river sands and crushed aggregates

The river sands has been applied for a long time for filtration process of municipal wastewater treatments and infiltration discharge of household wastewater treatments due to its good structural stability, chemical inert and infiltration behavior. But alluvial sands have limited source. The possibility of crushed aggregates application has been studied in several parameters which control the infiltration and purification behaviors as sand filter media. Comparing to the alluvial sand, the crushed aggregate as filter medium is rarely carried out. Crushed aggregate has been analogized with alluvial sand in terms of geometry characteristics and of biological treatment performance (Liénard *et al.,* 2001; Wanko *et al.,* 2005; Rolland *et al.,* 2009).

The crushed aggregate is well graded and less uniform (higher UC) (Gold *et al.,* 1992). The mineralogical composition shows that the crushed aggregate contains higher limestone content (Wankoet *al.,* 2004). Trails in France, 21 alluvial sands and 5 crushed aggregates have been compared in terms of their granulometric parameters and hydraulic behavior. Later, similar tests have been carried (Liénard *et al.,* 2001; Wanko *et al.,* 2004). The particles shapes of the alluvial sands and crushed aggregates are also different: the grains of crushed aggregates exhibit higher angularity and higher roughness (Tsomokos & Georgiannou, 2010).

The differences in the physic and composition of the two types of materials can induce the differences in biological treatment efficiencies. By comparing the treatment efficiencies of synthetic wastewater with the alluvial sand and crushed aggregates of similar effective diameter, Wanko *et al.,* (2005) showed that no significant difference in organic removals using the crushed aggregates as filter media if the granulometry carefully chosen. On the contrary, another study showed that crushed aggregates exhibited low efficiencies than river

sand due to the differences in particle shapes (Schäfer *et al.*, 1998). Thus the influences of sand type on the filter performance are still not quite clear.

I.2.5. Mineralogical characteristics of river sands and crushed aggregates

The filter materials are composed by various minerals. The compositions of most minerals are dominated by silica (SiO_2), lime (CaO) and alumina (Al_2O_3), and other major oxides of sodium (Na_2O), potassium (K_2O), iron (Fe_2O_3), magnesium (MgO) and titanium (TiO_2). The silica content varies widely from less than 50% to more than 99%. Even pure quartz sand may be bound with chemical cement, such as calcite. Comparing to alluvial sands, the crushed aggregates contain more calcites (Collis *et al.*, 1985). The composition is as function of the weather, the grain size and the source of the sand. For example, the silica content decreases with the grain sizes (Pettijohn *et al.*, 1973).

I.3. Mechanisms involved in vertical filtration systems in unsaturated conditions

The complex environment in the unsaturated filtration systems involves physical, chemical and biological phenomena, not only the filtration process but also a biological contact process. During the passage of feeding water, dissolved matters transported by liquid phase are uptaken by fixed biomass or free bacteria, or carried along with dynamic phase, or sorbed and settled in immobile phase; particulate matters are brought into contact with the surface of sand grains. Inert matters are retained on the surface or blocked in the pore throat, undergo the biological degradation and then converted into simpler forms for subsequent removal.

I.3.1. Solute transport mechanisms in porous media

The septic effluent discontinuously dosed on the filter surface, percolates slowly through the filter medium and the unsaturated conditions provide the presence of three phases: air, liquid and solid phase. The unsaturated conditions result in complex interaction, increase retention time, and facilitate aerobic bacteria growth (Kristiansen, 1981a; Boller & Kavanaugh, 1995).

I.3.1.1. Water saturation

The water flow can be described by Darcy's law as function of the gradient of the matric pressure and the hydraulic conductivity:

Equation 1: $$\vec{q} = -K(\theta)\nabla H = -\frac{K(\theta)}{\rho g}\left(\frac{d\psi}{dz} + \rho g\right)$$

The hydraulic conductivity is filter media attitude to transmit the water, depends on the pore space, which is severely impacted by the grain sizes, shapes and connected channels of the filter media. For an unsaturated media, the conductivity depends strongly on water content θ: as water content decreases, fewer pores are filled to conduct water and the paths of water flowing through the medium become discontinuous and tortuous, so the medium is less conductive. Thus, under the unsaturated conditions, the hydraulic conductivity K is a function of water content θ. According to van Genuchten (1980), the effective water saturation S_e varies from 0 to 1 and can also be expressed by the capillarity:

Equation 2: $$S_e = \frac{(\theta - \theta_r)}{(\theta_s - \theta_r)} = \frac{1}{(1+(\alpha\psi)^n)^m}$$

With: θ_r and θ_s represent respectively the residual and saturated water content; ψ represents the capillary pressure; α, n and m are shape parameters.

I.3.1.2. Theoretical approach of solute transport in porous media

The classical theories of solute transport in saturated or unsaturated porous media are based on the hypothesis: the solute transport takes place only in the liquid phase. The transport of non-reactive solute into a porous medium is quantified by two terms: 1) Convection (average velocity of flow) and 2) Dispersion. When a solution passes through a porous media at high velocities, molecular diffusion can be overlooked. In the cases of slow filtration, both the two mechanisms can be significant to solute transport behavior.

1) Convection: the inert solutes (concentration: C [ML^{-3}]) are transported generally by water flow which is described by Darcy velocity q as mentioned as Darcy's law;

2) Dispersion: the dispersion includes molecular diffusion and mechanical dispersion. The migration of solutes by molecular diffusion is caused by Brownian motion due to their thermal energy. The mechanical dispersion is resulted from the microscopic fluctuation of advection velocity.

The one dimensional equation of non-absorbing, non-degradable solute transport in a homogeneous unsaturated porous media generally accepted is Convection-Dispersion Equation:

Equation 3: $$\theta \frac{\partial C}{\partial t} + \theta v.\nabla C - \nabla.(\theta D.\nabla C) = 0 \text{ and} v = \frac{q}{\theta}$$

With θ is water content; q is Darcy velocity [LT^{-3}] and D is the dispersion coefficient of Fick's Law [L^2T^{-1}] which takes account both mechanical dispersion and molecular diffusion.

In the unsaturated flow environment, it is accepted that an immobile liquid phase exists, and this immobile phase does involve in the process of solute transport (van Genuchten & Wierenga, 1977). The ratio between the mobile and immobile liquid phase depends on the flow velocity. The two domains convection-dispersion equation is expressed by the following two equations with solute exchange process between the two regions:

Equation 4: $$\theta_m R_m \frac{\partial C_m}{\partial t} + \theta_{im} R_{im} \frac{\partial C_{im}}{\partial t} = \theta_m D_m \frac{\partial^2 C_m}{\partial z^2} - \theta_m v_m \frac{\partial C_m}{\partial z}$$

Equation 5: $$\theta_{im} R_{im} \frac{\partial C_{im}}{\partial t} = \alpha(C_m - C_{im})$$

The retardation coefficient caused by sorption process R is added to mobile and immobile phase. The water content θ is the sum of mobile and immobile water: $\theta = \theta_m + \theta_{im}$. Later, authors have claimed that the liquid domain in unsaturated porous media consists of the mobile zone, the stationary zone comprising the irreducible water content and an immobile liquid which moves very slowly (Kartha & Srivastava, 2008).

I.3.2. Particulate matters transport in porous media

Besides the desolved minerals and organic matters, particulate and colloidal matters are also transported with the feeding water flow. As regards to the retention by filter media are colloids and suspended small solids. The particles are brought into contact with sand grains (collectors) could be intercepted by 1) straining (or screening); 2) sedimentation; 3) physico-chemical factors and 4) diffusion (Huisman & Wood, 1974).

1) Straining: the particulate matters larger than the pore throats are either intercepted at the surface of the filter or blocked in the pore throat (McDowell-Boyer *et al.*, 1986);
2) Sedimentation: this process is important only for particles larger than 10µm. The settling action within the pores is comparable to conventional settling tank where the process of sedimentation is generally described by Stocks regime (Elimelech, 1995);
3) Multi-particle bridging: the bridging takes place when multiple particles interact and form a particulate arch against a pore throat that is larger than single particle. The likelihood of bridging depends on particle shape and the number of available particles in the pore throat (Santos & Barros, 2010);
4) Mass attraction, electrostatic interactions and diffusion: these mechanisms act on very small particles. The electrostatic forces play an important role to colloidal particles and their attachment process due to charged surface for both colloids and grains. The diffusion process resulted from Brownian movement brings particles into contact with containing surface and acts independently of filtration rate throughout the whole depth of filter medium, even when water is not flowing.

I.3.2.1. Macroscopic equations

The deposition of particulate matters causes the flow head loss and permeability damage. The macroscopic convection dispersion equations and kinetic equation have been proposed in order to integrate different mechanisms impacting the transport process of particles or colloids. These studies have been performed mostly in saturated porous media, but the unsaturated porous media has become a highlight since the gas-water interface has been noticed.

This macroscopic transport model accounts Brownian diffusivity into convection dispersion equation (Tien, 1989):

Equation 6: $$\frac{\partial C}{\partial t} + \vec{v}\nabla C = \nabla(D\nabla C + mC\nabla\emptyset\psi)$$

The term φ describes the colloid interaction energy and m describes the particle mobility which is related to Brownian movement by the following expression:

Equation 7: $$m = \frac{D_{BM}}{k_B T},\ D_{BM} = \frac{c_s k_B T}{3\pi\mu d_p}$$

The radial movement of non-Brownian particles and axial diffusion are overlooked comparing to the axial movement (flow direction), the Convection dispersion equation can be expressed:

Equation 8: $$-u\frac{\partial C_p}{\partial z} = \frac{\partial}{\partial t}(\varepsilon C_p) + \frac{\partial \sigma}{\partial t}$$

The term $\partial\sigma/\partial t$ is usually called filtration rate and σ is the concentration of retained particles. The filtration rate depends on the amount of particle available for deposition (C_p: concentration of particles in the fluid) and the deposit concentration (σ: particles that have already deposited). σ varies during the course of operation and with spatial coordinate. Thus the filtration rate is a time dependent local function.

I.3.2.2. Presence of air-liquid interfaces in unsaturated environment

The unsaturated porous media contains three phases: air, water and solid, and the interactions mostly occur at the solid-water interfaces (SWI) and the air (or gas) - water interfaces (AWI or GWI) (Schijven & Hassanizadeh, 2000). The particles are initially transported by liquid flow. When they move close to the AWI, they slow down due to the restricted flow around the air bubble. Once the colloids are near the AWI, they are attracted by the attractive interactions or through direct collision and finally attached to the AWI (Sirivithayapakorn & Keller, 2003). Torkzaban *et al.* (2008) showed, in their study of quartz sands, that the colloids retention increases with lower water flow velocity, lower water contents and higher ionic strength. These authors also indicated that neither the SWI nor the AWI dominates the colloids retention, but the straining remains the primary mechanism.

I.3.3. Hydrodynamic in unsaturated porous media: residence time distribution

❖ *Definition*

A flow of matters enters in a reactor, is composed by different fractions. The residence time of one fraction equals to the necessary time to run through the distance between the entrance and the exit. The matters can be simple molecular or aggregates of matters of a size more or less important, and all the particles of same fraction have identical residence time in the reactor. However, when a flow of matters crosses the entrance of reactor, different fractions are not crossing at the same instant. Because of this, the time passed of different fractions inside the reactor is variable. This phenomenon is represented by residence time distribution (RTD) (Danckwerts, 1953; Villermaux & Van Swaaij, 1969).

Considering a system with a constant volume *V*, the permanent regime is established with a flow rate *q*. The average concentration of concerned matter cross the outlet section is expressed as $C_y(t)$, and the mass has stayed inside during the time interval *dt*(from *t* to *t*+*dt*) can be expressed as $qC_y(t)dt$. The pollutant hydraulic residence time distribution can be described by the following function:

Equation 9: $$E(t) = \frac{qC_y(t)}{\int_0^\infty qC_y(t)} \text{and} \int_0^\infty E(t)dt = 1$$

The RTD can be considered as the responds of instant injection of an ideal tracer. This function is often transformed in Laplace field:

Equation 10: $$G(s) = \frac{q}{M}C_y(s) \text{and} G(s) = \int_0^\infty E(t)e^{-st}dt$$

Considering the moments of a residence time distribution are a number series of order *n*, the any moment of order *k* is represented by the product of convolution of the function *E(t)* and the function t^k:

Equation 11: $$\mu_k = \int_0^\infty t^k E(t)dt$$

The mean residence time is defined as the moment of the first order:

Equation 12: $$\bar{t} = \int_0^\infty tE(t)dt$$

The dispersion of time distribution around the mean residence time is defined as the variance:

Equation 13: $$\sigma^2 = \int_0^\infty (t - \bar{t})^2 E(t) dt$$

❖ *Hydrodynamic models*

The hydraulic residence time (HRT) in the filter medium affects numerous treatment efficiencies of sand filtration system. A different HRT means a different interaction time between pollutants and biomass. Residence time distribution varies with the hydraulic load, feeding-drainage frequencies, amount of the biomass present and the intrinsic characteristics of the packing materials.

The measurements of hydrodynamics are directed at two features of ideal flow conditions: plug flow and continuously stirred tank reactors (CSTR). Under plug flow conditions, the concentration-time distribution is simply a spike with a very small standard deviation about the nominal mean residence time; and for continuously stirred flow conditions, the distribution takes the form of an exponential function where the effect of flow dilution in steady flow conditions progressively reduce the concentration at the outlet. The circulations take place in the real reactors can be more complex with the presence of exchanges, retard or dead zones. Many attempts used a series of CSTR with dead zone, but the model did not always fit the observed RTD (Wen & Fan, 1975; Riemer & Harremoës, 1978).

In certain media, especially under unsaturated flow conditions, an immobile and a mobile zone exist at the same time and exchanges take place between the two phases, and this exchange is the main cause of the single dragging. By introducing volume parameters θ_{im} and θ_m representing the two zones (Van Swaaij *et al.*, 1969) , the tracer mass assessments on the two phases are established:

Equation 14: $$\frac{\partial C_m}{\partial t} + \frac{\theta_{im}}{\theta_m}\frac{\partial C_{im}}{\partial t} = D_z \frac{\partial^2 C_m}{\partial z^2} - \bar{u}\frac{\partial C_m}{\partial z}$$

Equation 15: $$\theta_{im}\frac{\partial C_{im}}{\partial t} = k_M(C_m - C_{im})$$

The k_m [T^{-1}] is transfer coefficient. Considering the system is closed at two extremities, the RTD function is given in Laplace transform (Sardin *et al.*, 1991):

Equation 16: $$G(s) = \frac{4\omega \exp\left[\frac{Pe}{2}(1-\omega)\right]}{(1+\omega)^2-(1-\omega)^2\exp(-Pe\omega)}$$

Equation 17: $$\omega = \sqrt{1 + 4t_m \frac{s[1+M(s)]}{Pe}}$$

Equation 18: $$M(s) = \frac{K_{im}}{1+st_M} \text{and} Pe = \frac{uH}{D}$$

Pe is defined as the Péclet number with H: the length of media, u: the flow velocity of mobile phase and D: the dispersive coefficient of mobile phase.

In wastewater reactors with fixed biomass, the RTDs in such systems usually present an asymmetric behavior called “tailing” due to the tracer exchange within the biofilm by diffusion (Riemer *et al.*, 1980; Stevens *et al.*, 1986). Under this circumstance, the biodiffusion models were developed by Riemer *et al*, (1980) considering that the transport phenomena exist in the phase of liquid flow are: convection, axial dispersion and molecular diffusion into the biomass and only the diffusion exists in the biomass section, summarized in the following table (Table1.4):

Table 1.4 : Differential equations and parameters of biodiffusion model

Transport phenomena	Liquid phase	Biofilm phase
Dispersion:	$K_1 K_4 \frac{\partial^2 c}{\partial y^2}$	-
Convection:	$K_4 \frac{\partial u}{\partial y}$	-
Molecular diffusion:	$K_2 K_3 K_4 \left(\frac{\partial u}{\partial \zeta}\right) \zeta = 0$	$\frac{\partial u}{\partial \vartheta} = K_2 K_4 \frac{\partial^2 u}{\partial \zeta^2}$
Coefficients		
c:	Ratio between the actual tracer concentration and input tracer concentration	
u:	Ratio between the tracer concentration in biofilm and input tracer concentration	
ζ:	Distance in the biofilm from biofilm surface	
K_1:	$K_1 = \frac{D}{vH} = \frac{1}{Pe}$	Inverse of the Peclet number
K_2:	$K_2 = \frac{HD_B}{vL^2}$	Ratio between the actual retention time and the characteristic time for molecular diffusion into biofilm
K_3:	$K_3 = \frac{L\omega}{\varepsilon}$	Geometric constant with ε is the actual porosity
K_4:	$K_4 = \frac{\varepsilon_v}{\varepsilon} = \frac{T_{hv}}{T_h}$	Ratio between the water volume without biofilm and actual water volume

Based on the similar theory, Lakel et *al.* (1998) gave the resolution in Laplace domain as a function *G(s)* for a pulse input:

Equation 19: $$G(s, f, Pe, \eta) = \frac{2R * e^{Pe}}{e^{Pe/2}\left[\frac{Pe}{4} - \frac{R^2}{Pe}(e^{-R} - e^{R}) + e^{R}\left(\frac{Pe}{2} + R\right) - \frac{Pe}{2} + Re^{-R}\right]}$$

R is expressed by following equation:

Equation 20: $$R = (Pef)^{1/2}\left(s + \frac{\eta}{f} + \frac{Pe}{4f} - \frac{\eta^2}{\frac{f(1-f)}{s+\eta(1-f)}}\right)^{1/2}$$

And:

Equation 21: $$\eta = \frac{k_e a_e H}{\bar{u} A_m} \quad , f = \frac{S_m}{S_{im}+S_m}$$

With k_e: the exchange coefficient, and S_m, S_{im}: the section of mobile and immobile zone.

I.4. Treatment efficiencies and functioning of vertical drained packed filter

The filtration reactor functions as an aerobic, fixed biomass reactor. The mechanisms of pollutants removal involve physical, chemical and biological aspects. The eliminations of pollutants and pathogenic germs depends on the characteristics of materials and the states of filter functioning. Infiltration and purification capacities evolve with the development of clogging which has two actions on the filter: favorable to biological treatment by biofilm and unfavorable due to overly accumulated matters (SS from feed water + biofilm developed in the filter). This is a complex process with various interactions among the feed water input, the secretion of biofilm and the surface of sand grains. In Figure 1.2, some current facts of sand filter functioning is presented in an overview schema.

I.4.1. Mechanisms of retention and/or degradation and purification

❖ *Suspended solids*

The elimination of suspended solids depends on the particle sizes and the pore space of the sand bed. The mechanical retention takes place when suspended particles are larger than the pore throats; smaller particles could be removed by the interception and the adsorption. (Gougoussis, 1982; McDowell-Boyer *et al.*, 1986).

❖ *Organic matters*

The organic pollutants represented by organic carbon can be particulate or soluble, inert or readily biodegradable. The particulate organic matters retained on the surface of the sand massive undergo the hydrolysis and become less complex or soluble which can be readily assimilated. The soluble organic matters are retained at various depths. The complete mineralization is achieved by the aerobic biodegradation under the actions of heterotrophic bacteria which are fixed in biofilm. Under the anaerobic conditions, organic matters are only incompletely, slowly degraded into organic substances easily degradable such as proteins and also some polymers difficult to degrade such as celluloses. The degradation sometimes leads to the formation of methane, hydrogen, sulfide…(Lefevre, 1988; Rodgers *et al.*, 2005).

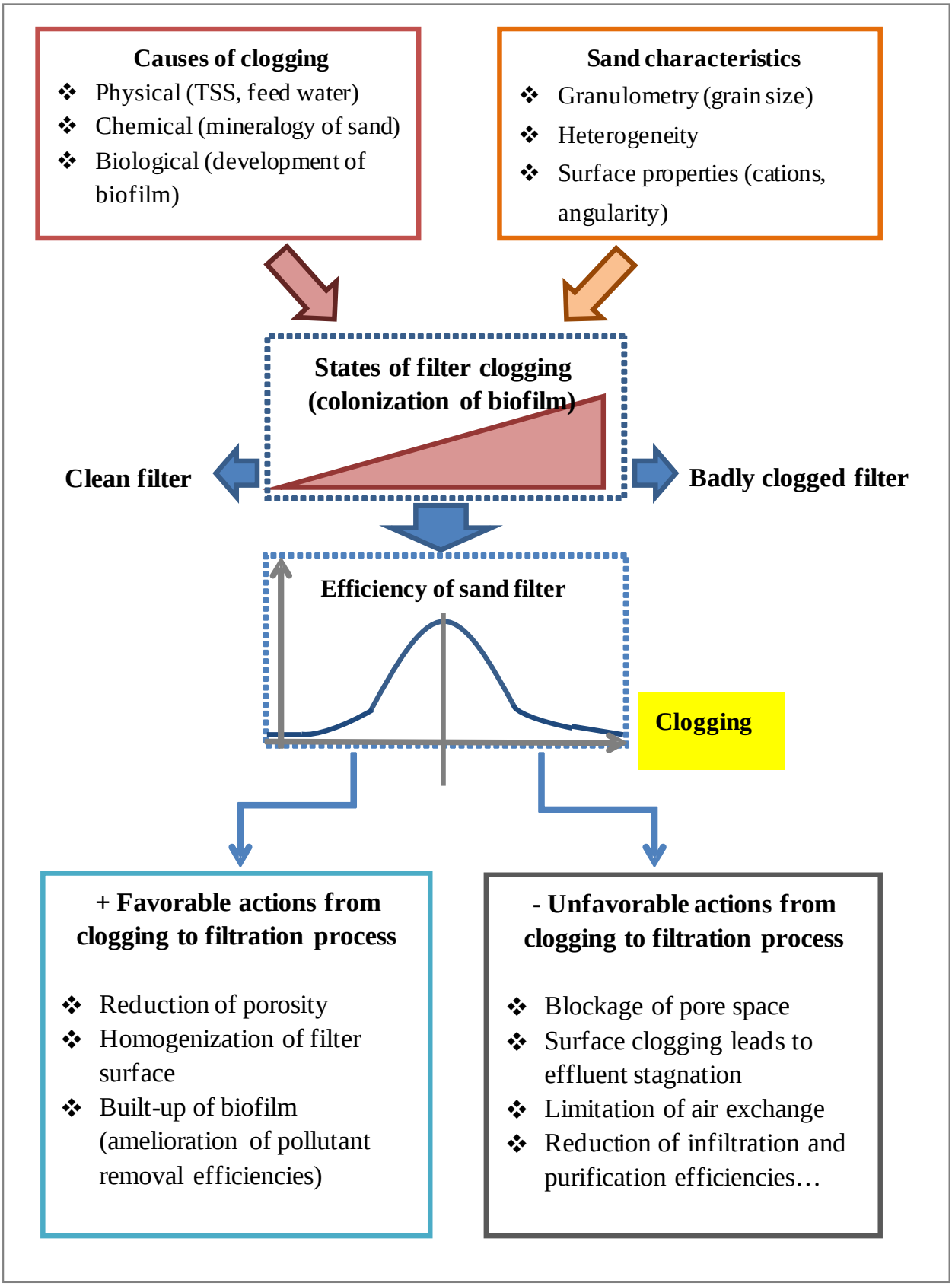


Figure 1.2 : Overview of current knowledge on sand filter functioning

❖ *Nitrogen*

The nitrogen pollutants in septic effluent are mainly composed by ammonia in cationic form ($N\text{-}NH_4^+$) and organic nitrogen which can be retained in the mineral massive by adsorption and ammonification. The removal of ammonia is dominated by the nitrification under the aerobic reactionsbyautotrophic bacteria from Nitrobacteriaceaefamily: *Nitrosomonas*(1) and *Nitrobacter* (2):

(1) Nitritation: $NH_4^+ + {}^3/_2\, O_2 = NO_2^- + H_2O + 2H^+$

(2) Nitration: $NO_2^- + {}^1/_2\, O_2 = NO_3^-$

The nitrification can be quite performing for the cases of filtration systems because the oxygen is not limited. The authors indicated that for a hydraulic loading around 5 cm/day, a delay of latency about 19 days was necessary for establishing the fauna of Nitrobacteriaceae and after this latency, the explosion of Nitrobacteriaceae has been found between the surface and the depth of 12cm (Ardakani *et al.*, 1974). For a strong loading, the oxidation from nitrites to nitrates becomes incomplete since the oxygen is not enough. The aeration of the filtration milieu is the necessary condition for the nitrification.

The process of denitrification exists in two ways: the NO_3^- and NO_2^- can be reduced chemically or biologically into the mix of NO, N_2O, N_2. The denitrification may take place under following conditions: the establishment of nitrification, the existence of anoxic zones in the sand massive, the basic pH and enough organic substrate to maintain the bacterial activities. Under these strict conditions, by comparing to the nitrification, the infiltration systems are less promising of denitrification. Many families of bacteria are able doing nitrate respiration via the nitrate reductase A enzyme:

Denitrification: $2NO_3^- + 12H^+ + 10e^- = N_2 + 6H_2O$

❖ *Phosphorus*

Low removal efficiencies of phosphorus pollutants (85% orthophosphates and other on organic forms) are dominated by adsorption, precipitation and short-term biological immobilization. The adsorption of PO_4^{3-} is mainly linked to the nature of the filtration massive which is optimized by higher clay, calcareous, hydroxides of iron and aluminum contents of the materials and basic pH environment; the precipitation is related to the ionic composition of applied effluent: the presence of Fe^{3+}, Al^{3+}, Ca^{2+} favorites the precipitation of PO_4^{3-}; the phosphorus can shortly stabilized by biological immobilization (Gougoussis, 1982; Zanini *et al.*, 1998; Robertson, 2003).

❖ *Microorganisms*

The microorganisms behave like other particles in the filtration massive where several mechanisms can involve: sedimentation, filtration, adsorption, predation… The major processes of bacterial elimination in the sand filter are the filtration, adsorption and microbial degradations which include the predation, the nutritive competition, and the parasitism (Lefevre, 1988; Gammack *et al.*, 1992; Chabaud *et al.*, 2006). The viruses are so small that the elimination by filtration cannot be effective enough, but the adsorption and inactivation can be involved. The attenuation of bacteria and virus varies from case to case, and depends on various factors such as the pH, the temperature, the gas-water interface which is quite noted in unsaturated porous media (Wan *et al.*, 1994).

I.4.2. Influencing factors of treatment efficiencies

Both environmental conditions and operating parameters impact the pollutant removals by sand filtration. Numerous studies were conducted by the alternation of the filter design in order to optimize the filter performance, such as daily hydraulic loads, dosing frequencies, the presence of plants and types of materials. The performance is an interactive, dynamic process between the hydraulic and purification behaviors. Some studies are summarized in Table 1.5:

Table 1.5 : Studies in purification efficiencies of sand filters and influencing factors

Analyzed parameter	Influencing factors	Reference
TSS	❖ **Grain size:** larger grain size results deeper retention of solids	Ellis & Aydin, (1995)
Organics	❖ **Presence of biofilm growth:** induces higher organic removals at initial state of sand columns; ❖ **Depth of filter media:** great depth of filter has higher removals;	Rodgers *et al*, (2005) Lloréns *et al*, (2011)
Nitrogen	❖ **T°C:** nitrification is inhibited at low T°C; ❖ **Presence of clogging:** anoxic zone may induce the denitrification; slight clogging also improves the nitrification; ❖ **Organic loads:** higher organic loads reduces the activities of nitrifying bacteria; ❖ **Presence of plants:** in combination with nitrification;	Kristiansen, (1981 II) Van Cuyk *et al.,* (2001) Rodgers *et al.*, (2005) Gill *et al.*, (2009) Lloréns *et al*, (2011)
Phosphorus	❖ **Mineralogy of filter media and the clay contents:** both impact the P removal; ❖ **Reactivity of filter media:** alkaline materials under low organic loads results better P removal;	Gill *et al.*, (2009)
Microorganisms	❖ **Hydraulic and organic loads:** under unsaturated conditions, no effect of hydraulic loads on the removal; higher organic loads leads to higher bacteria removal ❖ **Presence of slight clogging:** the utilization of the media increased resulting in the improvements of removals; ❖ **Distribution mode:** uniform pressure dosing results better removals; ❖ **T°C (seasonal operation):** virus removals diminished during colder weather	Van Cuyk *et al.,* (2001) Ausland *et al.,* (2002) Kauppinen *et al.,* (2014)

By reviewing previous studies, the purification efficiencies of a packed filter often undergo the influences of several factors at once, but the hydraulic retention time seems to be the governing factor. The traditional single pass filters packed with grained materials (soil, gravel, sand…) show less effective capacities in total nitrogen and phosphorus removals (about 30% of Total N and unstable removal of P) which are predictable under the aerobic filtration conditions. The filtration system will also clog, and their performances are considered being affected.

I.4.3. Causes of clogging (total suspended solids + biofilm) and impacts of clogging on the filter performance

The filter media clogging has been recognized as common and significant problem that affects the hydraulic and treatment efficiencies of the system, especially the long-term performance(Kristiansen, 1981a; Siegrist & Boyle, 1987). The clogging consists of the obstruction of the pore space, and it tends to appear in the upper layer of the sand filter where the substrates and microorganisms are most abundant (Rodgers *et al.*, 2004). The causes of clogging can be related to various factors, the environmental and operational conditions both influence the process. By the nature of these mechanisms, the clogging is generally categorized as physical, chemical and biological process (biofilm) (Baveye *et al.*, 1998).

I.4.3.1. Causes of clogging

The occurrence of clogging is related to physical, chemical and biological process. The clogging development is complex because these processes take place simultaneously, continuously and interact with the substratum and also among the aggregates themselves.

- *Physical clogging (origin of feed water)*

The deposition of inorganic and organic solids at the surface develops into a physical clogging. Feeding waters with high concentration of suspended solids have been frequently observed to lead to a severe clogging through the process of filtration (Rice, 1974). The organic particulate matters difficultly or non-biodegradable can also accumulate inside the pore throat causing pore blockage (Siegrist & Boyle, 1987; Nguyen, 2000). Under high loading conditions, this accumulation of partly hydrolyzed organic particle matters reduces more rapidly the pore space than the development of biofilm (Zhao *et al.*, 2009).

- *Chemical clogging (origin of feed water + filter media)*

The chemical clogging is mainly caused by mineral precipitation in filter media due to the chemical properties of media grains, mineral components of wastewater, biochemical activities of biomass, and environmental factors like temperature and pH that are able to modify the solubility of these minerals (Baveye *et al.*, 1998). The most commonly observed precipitates are $CaCO_3$; Fe, Al or Mn hydrous oxides; phosphate precipitates; and sulfate, sulfide precipitates. The carbonate and phosphate precipitates highly depend on the calcareous

content and pH value of filter. In alkaline and calcareous environment, the free calcium in the material is responsible for the high correlation between calcium and phosphorus in phosphate minerals, and the high pH value results in an increase in CO_3^{2-} concentration, allowing or accelerating $CaCO_3$ precipitation (Cooke *et al.*, 1999). Organic matters in septic effluent cause irons in non-calcareous sites to become soluble and subsequently results in Al-P precipitation coating on the surface of sand grains below in the filtration beds (Robertson, 2003). The formation of sulfide is generally microbial catalyzed. The sulfate reducing bacteria "breathing" sulfate rather than oxygen are known to oxidize incompletely a number of organic acids and alcohols into acetate where sulfates act as electron accepters with sulfides as by-products. The ferrous iron (Fe^{2+}) existing already or resulting from bacteria activities readily associates sulfides and forms into FeS which are precipitates as black colloids and has a very low solubility. The FeS colloids bond strongly with organic matters, humus and do not seem to accumulate on mineral soil particles, but they form a black layer in clogged filter media (Kristiansen, 1981a; Gottschalk, 1986). The chemical clogging is less pronounced than physical clogging.

- *Biofilm*

The biological clogging mainly consists of biofilm which leads to the decreasing of pores space results in clogging. Net-work surrounding the microcolonies with extracellular polymers substances (EPS) (Kim *et al.*, 2010) by bacteria adhered to material grains is the main cause of the biological clogging (Rodgers *et al.*, 2004a). The biofilm plays an important role on the colonization ability of bacteria to solid surface to protect themselves from environment in order to survive (Costerton *et al.*, 1981; Ronner & Lee Wong, 1998; Van Cuyk *et al.*, 2001; Mauclaire *et al.*, 2002).

I.4.3.2. Influencing factors on clogging process

The clogging process is impacted by various factors which can be operational, environmental and of bacteria themselves: such as materials characteristics, feeding water characteristics, and surface properties of bacterial stains. The interactions are especially noticed between the material surface and the particulate or colloids matters and the microorganisms. Some factors are summarized in the Table 1.6:

Table 1.6 : Factors influencing clogging process

Influencing factor	Mechanisms of impact	Reference
Organic content (feed water)	Higher organic content in feed water leads to higher microbial activities and EPS production	Reneau *et al.*, 1989; Liu *et al.*, 2003
Grain size (filter media)	Grain size impacts directly the physical clogging and also the biofilm development at higher organic content	Kristiansen, 1981a
Surface roughness or angularity of sand grains (filter media)	Rough surfaces might be favorable of bacterial deposition thus biofilm	Jacob *et al.*, 2007 Wanko *et al.*, 2005
Hydrophobicity of cell surface (bacteria species)	Bacteria with hydrophobic surface are easier to adhere to sand surface	Jacob *et al.*, 2007

From the above table, the filter media has great impact on the development of clogging, not only on the physical process (size of particulates vs pore space), but also on the bacteria adhesion and further biofilm formation (cell surface vs sand grain surface). The process of clogging is rather complex, and the impacts of material properties are not quite clear.

I.4.3.3. Impacts of clogging on the filtration and treatment performance

As mentioned in the overview schema of sand filter functioning (Figure 1.1), the actions brought by clogging to the filtration and purification process can be favorable and unfavorable. The transition between the two actions is a dynamic process involving mainly the overly accumulation of particulate matters and biofilm.

❖ *Favorable impacts*

The presence of slight clogging generated by the accumulation of biomass and particulate matters favorites the filter performance at the early stage of operation (Okubo & Matsumoto, 1979). The pore size in the upper layer is partially reduced by the particulate matters straining and the surface coating on the grains, and the network among these retained matters and newly generated biomass benefit to achieve more uniform infiltration (Kristiansen, 1981c). As mentioned, the hydraulic retention time plays an important role in purification process. A slight clogging reduces the initial hydraulic conductivity thus hydraulic residence time (HRT) creases comparing to the "clean" bed which increases the chance of pollutant adsorption, exchange, biodegradation of organic carbon by heterotrophic bacteria and bacterial adhesion (Healy *et al.*, 2007). Furthermore, the microbial communities (nitrifying and denitrifying bacteria) developed in sand filter are also responsible for the biotransformation of the nutrient pollutants (Pell *et al.*, 1990). The local denitrification conditions sometimes can be satisfied beneath the surface biomat zone where the environment is enriched in organic carbon with less content in dissolved oxygen (Gill *et al.*, 2009). When an excessive surface crust has formed, the terminal head loss can occur. In the study of (Campos *et al.*, 2002), the sand filter with higher developed biomass did not show the improvement in TOC removals in the effluent.

❖ *Unfavorable impacts*

The clogging is often recognized as problematic functioning deficiencies and observed in laboratory sand columns or onsite sand (or soil) filters (Siegrist & Boyle, 1987). The functioning deficiencies include purification difficulties that usually companied by hydraulic difficulties over time, such as: the influent ponding, the decreased infiltration rate (field study), the hydraulic head loss and the decreased hydraulic conductivity (Rice,1974; Kristiansen, 1981a; Mays & Hunt, 2004; Beach *et al.*, 2005). The loss of initial infiltration rate is inevitable. According to Okubo & Matsumoto (1983), under saturated conditions, the evolution of infiltration rate over time consists of three successive stages: (i) the aerobic period with a rapid decrease of infiltration rate companied with the reduction of dissolved oxygen, (ii) the transitional period (constant or slight increase of infiltration rate with low dissolved oxygen (DO) concentration) and (iii) an anaerobic period in which infiltration rate decreased rapidly until the clogging developed under anaerobic conditions. The clogging may

be delayed under unsaturated flow conditions. This decreased capacity is the result of the abatement of the pore size and of their interconnections or even of a complete filling of pores.

The influent ponding represents the serious threat to the use of the sand filter. When the ponding occurs, even not at the entire filter surface, some regions (especially near the inlet) can be under saturated conditions with reduced air exchange and limited dissolved oxygen contents. During the decomposition process of organic pollutants under the action of heterotrophic bacteria, the dissolved oxygen in the liquid phase is consumed, when the air phase in the medium fails to renew itself, the mineralization in aerobic way may be interrupted and even stopped. With the presence of severe clogging, the microflora becomes specific and less diverse in an environment poor in oxygen and rich in organic matter. The degradation mechanism alters to anoxic anaerobic, enriched in the carbon dioxide (CO_2), and the methane (CH_4). The lack of dissolved oxygen also blocks the nitrification. The accumulation in the pores of partially degraded organics and particulate matters enhances the clogging even more and the filter reaches the terminal use lifetime. The following scheme describes the development of clogging and the interaction with the hydraulic and purification behavior (Figure1.3):

The current knowledges of packed filtration beds suggest that the development of clogging is inevitable. More and more studies showed that the evolution of biofilm tends to be an important process since the suspended particulates are brought by the feed water and the precipitation has little impacts, but the biofilm develops around the material grains, and occupies more and more the pore throats until reaches the equilibrum. However, informations are lacking, espeicially on the evolution in time and depthes of biofilm compositions, their characteritics and quantities, which also the focus of this study. In the two following sections, an bibliographic overview revisits some key information of biofilm.

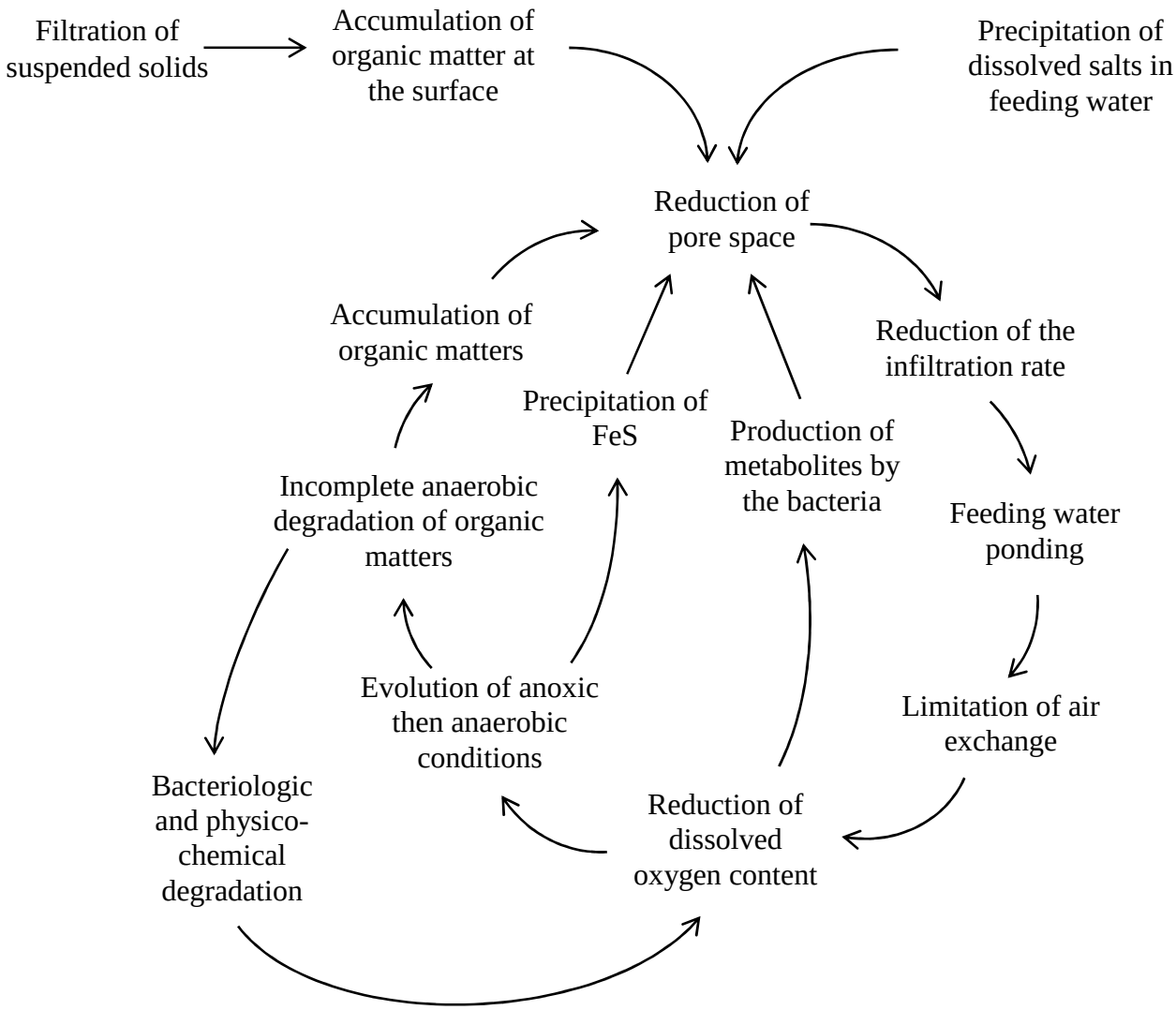


Figure 1.3 : Simplified schema of clogging development (Maillard, 1998)

I.5. Biofilm development

The survival of the biomass in filtration systems is to colonize the solid surface (ex: sand) and to form into biofilm. As mentioned in the last section, the biomass enhances the treatment efficiencies and also induces the clogging. This section focuses on development of biofilm and the main structures and characteristics.

I.5.1. Definition, characteristics and functions of biofilm

I.5.1.1. Biofilm definition and fixed bed systems

Biofilm is microbial derived sessile community characterized by cells that are irreversible attached to a substratum or an interface or to each other are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription. These microorganisms may undergo physiological and genetic modifications during their transition from planktonic mode of growth (Donlan & Costerton, 2002).

Compared to suspended microbial growth systems, wastewater treatment with fixed biomass (fixed bed or moving bed systems) has several advantages: increase of biomass residence time which results in lower space requirement and reduced hydraulic residence time, high active biomass concentration, as well as lower sludge production (Verma *et al.,* 2006). Fixed bed systems often use porous mediums that contain pores (or voids) and provide large specific surface for the development of biofilm: it can be natural materials (soils, sand, river sediments, aquifer zone…) or artificial mediums (crushed aggregates, glass or plastic solid materials…). In porous media, microbial cells in suspension may adhere firmly to solid surface that comprises the effective pore space. Under favorable conditions, adhered cells reproduce at the surface and increase the amount of attached biomass. The Figure 1.4 shows a biofilm coating on quartz sand grains:

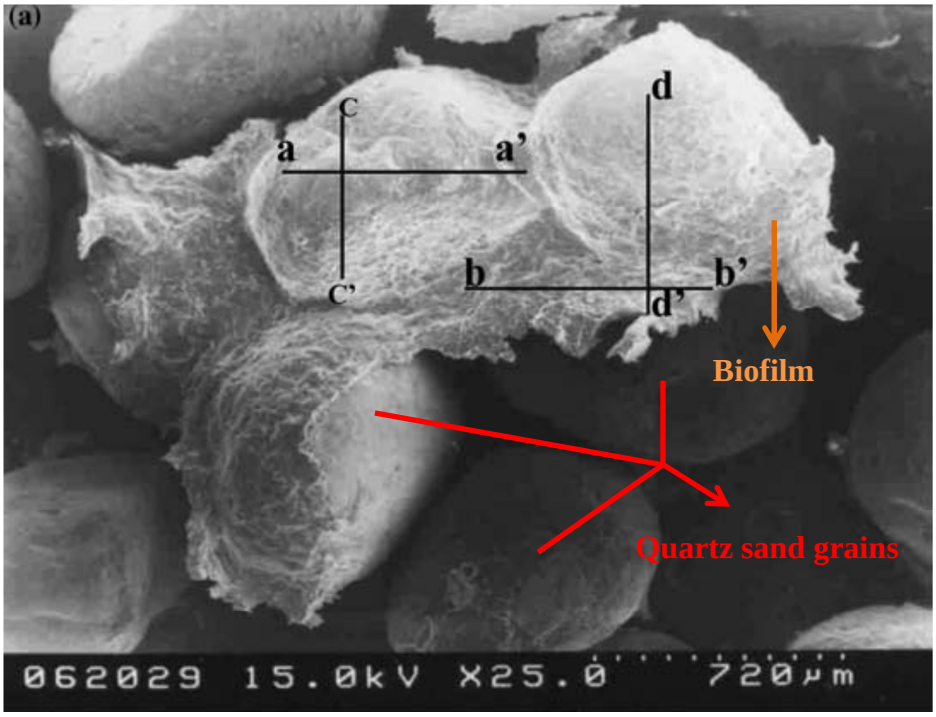


Figure 1.4 : 7 months growth of biofilm on quartz sand grains (Jean *et al.*, 2004)

I.5.1.2. Introduction of extracellular matrix of biofilm

Biofilm offers their member cells the benefits of the protection from environmental stress. The spatial distribution of biofilms presents various structures and sizes: monolayer biofilm of thickness of 5-25µm resulted from laboratory pure culture with single species (Andersson *et al.*, 2008); and biofilm of 40µm thickness in anaerobic wastewater treatment plant (De Beer *et al.*, 1994). In fixed biomass systems, biofilm is heterogeneous and organized by microcolonies structures including void space and water channels. These water channels or pore spaces within biofilms associate with bulk liquid and allow the transport of substrates including oxygen, and free cells, and the convey of metabolic wastes (Lawrence *et al.*, 1994; Massol-Deyá *et al.*, 1995).

This “gel like” structure of biofilm is mainly composed by the extracellular matrix of biofilm: EPS represented 50 to 90% of total organic matters of biofilm (Wingender *et al.*, 1999). The matrix of EPS is highly hydrated (90% water). The majority of EPS is composed by mostly macromolecular complex polysaccharides, proteins, small quantities of lipids, humic substance-like and nucleic acids (Flemming & Wingender, 2001a). The fraction of these macromolecules varies according to the type of microorganisms, cell physiological state and

environmental conditions: the quantity of polysaccharides and proteins represents 75-89% of the composition of EPS (Tsuneda *et al.,* 2003a). Minerals are also found trapped in the matrix and associated with the EPS (Landa *et al.,* 1997) and minerals can represented 10-48% of biofilm dry weight depending on the extraction methods (Bourven *et al.,* 2011; D'Abzac *et al.,* 2012).

I.5.2. Biofilm development steps

Before the biofilm formation, the bacterial cells attach themselves on the substratum surface, which start with the very first possible step: the formation of conditioning film. Having been observed in some medical biofilm cases, the conditioning film is realized by adsorbing macromolecules originated from bacterial metabolism and neutralizing the excessive free energy at the proximity between the cells and the solid surface (Marsh, 1995). The conditioning film has not been reported in cases of biofilm in filter media. The development of biofilm begins under favorable conditions, followed by several steps: cell proliferation, production of EPS, maturation and detachment. As shown in Figure 1.5, the development of biofilm is generally described as following stages (1-conditioning of the surface and reversible adhesion, 2-irreversible adhesion, 3, 4-proliferation and accumulation, 5-maturation and detachment):

- *Proliferation and accumulation*

Resulted from the step of irreversible adhesion, the initial coating of cells and EPS are established which favorites the growth of cells inside the initial film. The cells obtained from cells divisions form clusters or communities of bacteria. There is also the recruiting of free cells in liquid milieu. The heterogeneity of the biofilm increases during the accumulation and there is not necessarily only uniform layer but the colonization and architecture of different bacterial communities (Stoodley *et al.,* 2002).

- *Maturation and detachment*

Equilibrium state achieves between the production and accumulation of new cells and EPS and the detachment of the aged biofilm, and the quantity the biofilm reaches a limit value and maintains constant (Liu *et al.,* 2003). Besides the cells mortality, the cells derives inside the biofilm and certain biofilm constituents including the cells can be transferred and released back to the bulk liquid. The process of detachment is governed by biotic and abiotic factors:

metabolism, shear forces, and environmental stress. The shear forces of the biofilm detachment are erosion (losing small parts of biofilm caused by liquid flow), abrasion (caused by collision between the biofilm and small solid particles) and sloughing (losing large part of biofilm caused by brutal changes of environment).

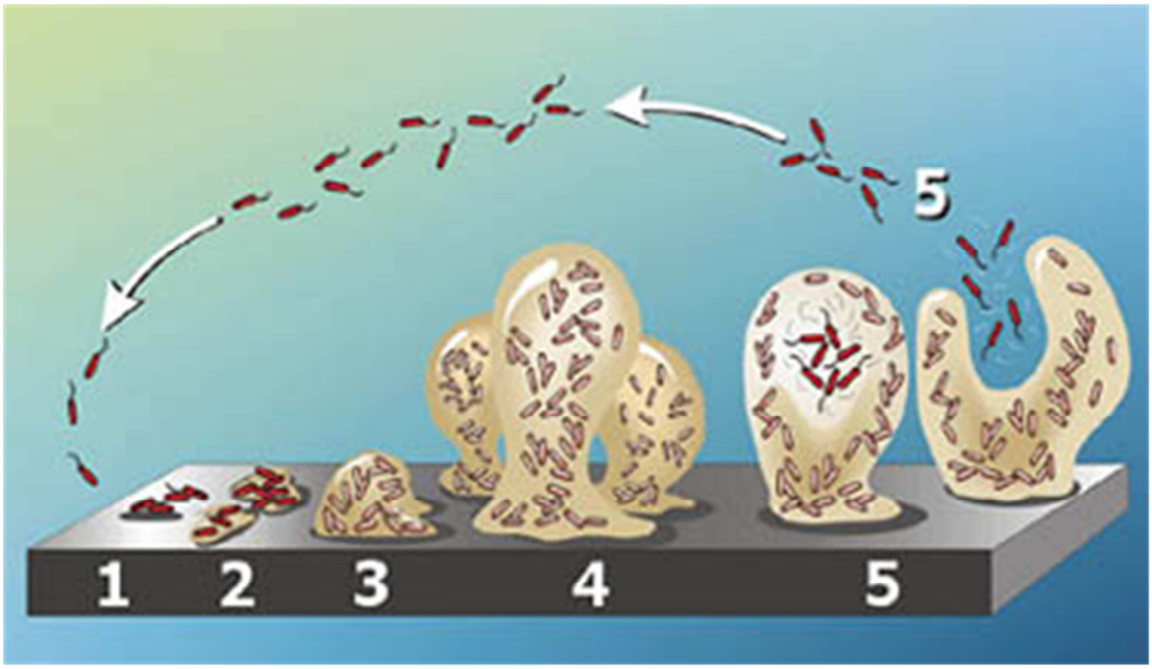


Figure 1.5 : A simplified description of the development of the biofilm onto a mineral surface
Resource: [Center for Biofilm Engineering Montana State University, 2003]

I.5.3. Bacterial adhesion

As to initial the solid surface colonization, bacterial cells have to approach and adhere to the surface. The process of bacterial attachment can be divided into two steps: reversible adhesion and irreversible adhesion. The process is governed by various interactions.

I.5.3.1. Initial stage: physical transport and retention

1) Hydraulic transport: convection-diffusion

The microorganism migration occurs in the bulk liquid and at the liquid-solid interfaces, as well as the air-liquid interfaces under the unsaturated conditions. Similar to the colloids, the bacteria are transported with liquid flow towards the solid phase. This step includes convection and diffusion transport. Higher flow velocity induced higher bacterial cell penetration or earlier breakthrough (Huysman & Verstraete, 1993; Sarkar *et al.*, 1994). Hydraulic loading rate and effective grain size impact directly the convection transport of bacteria by governing flow velocity and capillary force (Stevik *et al.*, 1999). Diffusion transport intervenes when Brownian movement cannot be neglected, especially when the porous media is under rest conditions (no loading) (Corapcioglu & Haridas, 1985).

2) Physical retention (Straining)

The straining is physical mechanism of movement blocking through of the bacteria larger than pores. The straining mechanism highly depends on filter media characteristics, degree of water saturation, bacterial cells and the development of clogging zone (Ausland *et al.*, 2002).

I.5.3.2. Reversible adhesion: physico-chemical stage

The reversible adhesion takes place when bacteria are close enough to the solid surface. This process involves the interactions between the natures of the two surfaces (solid surface vs. cell surface), under the chemical environment of the liquid phase. The reversible adhesion is based on the extended DLVO (XDLVO) (extended Derjaguin-Landau-Verwey-Overbeek) theory which includes three groups of interactions: generally attractive Lifshitz-Van der Waal interactions (LW), electrostatic interactions (EL) and Lewis Acid-base interactions (AB) (electron donating and receiving) (Van Oss, 1989). The attraction or repulsion of bacteria cells to the solid surface is described by total Gibbs energy which is the sum of these three interactions:

$$\Delta G = \Delta G^{AB} + \Delta G^{LW} + \Delta G^{EL}$$

When ΔG is negative, the attraction takes place and ΔG is positive, the repulsion occurs. The hydrophobicity of cell surface is another interaction playing important role in bacteria adhesion to solid surfaces.

1) Nature of material surface

Compared to the cell surface, the sand surfaces are more stable and uniform. The study of Jacobs *et al*., (2007) suggested that natural quartz sand particles have slightly hydrophilic negatively charged surface and the surface roughness may reduce the repulsion. A study of surface chemistry showed that natural quartz sand surface contains mainly the oxide of silica and iron, also presents weak contents of Ca and Al (Shani *et al.*, 2008).

❖ *Presence of multi- or divalent cations*

Divalent ions have shown to enhance the bacterial adhesion to surface by the compression of electrical double layer (EDL) (De Kerchove & Elimelech, 2008). Ca^{2+} ions in particular have shown to play an important role by involving in non-specific such as a neutralization of surface charge and reduce the repulsive energy barrier (Kuznar & Elimelech, 2004). Ca^{2+} ions can also act as bridging ions by forming the cationic bridges between the cell surface and solid surface (Rose *et al.*, 1993). The divalent ions can be brought by feed water and may be released by the sand medium.

2) Nature of bacterial cell surface (DLVO interactions + steric interactions)

The bacterial cell surface is a highly dynamic surface responding to environmental changes. Charged group may associate or dissociate upon pH, ionic strength of suspending fluid, and also upon the approach of a charged surface. Under most physiological conditions, bacterial cell surface carries a net negatively charge with a few exceptions.

❖ *DLVO interactions*

The LW interactions are always attractive and contribute the adhesion. In contrary, the electrostatic interactions are dominated and repulsive in a low ionic solution of pH 7 (I<0.001M) (Redman *et al.*, 2004). Today it is well known that increasing ionic strength reduces the EDL thickness and the cell may be brought close enough to the solid surface so that attractive LW interaction dominates (Rijnaarts *et al.*, 1999).

❖ *Steric interactions (non -DLVO interactions)*

Macromolecular-substratum interactions are generally called steric interactions which mediate both reversible and irreversible adhesion. The steric interaction can be repulsive or attractive, which depends on the hydrophobicity of cell surface and substratum (Rijnaarts *et al.*, 1995). Steric repulsion occurs when cell surface macromolecules are hydrophilic and have no affinity for the substratum (ex: glass is highly hydrophilic surface); on the other hand, if the macromolecules or parts of them have an affinity for the substratum (ex: Teflon is hydrophobic surface) and exceeding certain critical value, attractive bridging may take place. While at higher ionic strength, the shift from DLVO controlled adhesion to steric controlled adhesion is observed and the adhesion efficiency depends on the type of cell surface coating (Rijnaarts *et al.*, 1999). The nature of cell surface coating determines the complexity of steric interactions between bacteria and surface which may reduce the rate of deposition, but at the same time, may cause the irreversible adhesion (Tsuneda *et al.*, 2003). The Figure 1.6 shows the main interactions involved in the reversible adhesion.

Studies showed that the bacteria movements also depend on their electrophoretic mobilities. Following the definition given by Ohshima (1995), the bioparticles such as bacteria possessing the hard core and an adsorbed permeable gel-type layer are considered as soft particles, and exhibit the electrophoretic behaviors. The electrophoretic mobility of the soft particles is influenced by the interface properties and described by Ohshima formalism where the interphase between the soft particle and the outer medium is considered as homogeneous. Under the high ionic strength condition, this model suggests a finite of non-zero plateau that the electrophoretic mobility depends on the space charge density of the soft layer and the soft layer parameter and its dynamic viscosity. However the Ohshima's prediction deviates when the partial dissociation of microbial interphase charge becomes significant under low ionic strength condition (Duval *et al.*, 2005). Face with the limitations of Ohshima's model, the diffuse soft particle electrokinetic formalism was developed by Duval and Ohshima (2006): under decreasing ionic strength condition, the inhomogeneous of the polymer segments affects significantly the electrophoretic mobility and the interphasial diffuseness may evolve with the physical-chemical composition of the medium. The literature review of Duval and Gaboriaud (2010) combined both numerical and experimental data and allowed going beyond the classic approaches of impermeable particles and revaluating the

impacts of the chemical heterogeneities and physical diffuseness of soft particle interphase on the electrophoretic motions.

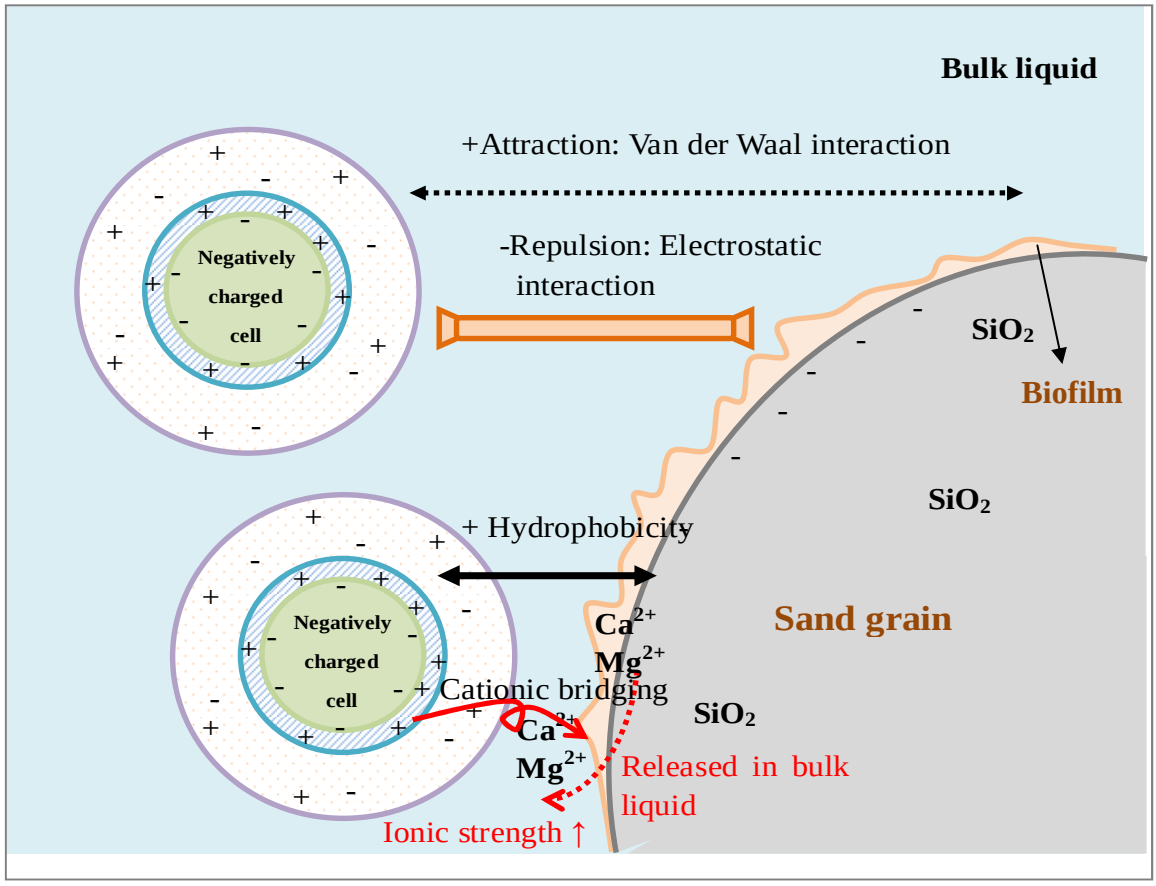


Figure 1.6 : Main interactions involved in the reversible adhesion

I.5.3.3. Irreversible adhesion: biochemical stage

Comparing to reversible adhesion, this stage is permanent between bacterial cells and solid surface involving biochemical phenomenon. The steric attraction promotes the irreversible adhesion. The cells presented on the surface attach themselves by intermediary of their metabolism activities, EPS and multivalent cations in EPS. The irreversible adhesion occurs through the implication of cellular appendices, such as adhesine (protein and polysaccharides) and/or the production of EPS that connect bacteria and adsorbent. This process less or more rapid depends on different types of microorganisms, environment and the contact time (Characklis & Marshall, 1990).

❖ *Presence of EPS*

The presence of EPS covering on a cell surface alters the physiochemical aspects of bacterial cell adhesion onto solid surface. The extracellular protein and amino acids increase the surface hydrophobicity thus favorite the attachment (Pell & Nyberg, 1989). The uronic acids enhance the anionic properties of some Gram negative bacteria and allow the association with the divalent cations to increasing the binding force (Molle *et al.*, 2005). Anionic polysaccharides and amphiphilic cell coatings both exhibit steric repulsion at hydrophilic and hydrophobic solid surface (Rijnaarts *et al.*, 1999). The Figure 1.7 and Table 1.7 summarize the interactions involved in the bacterial adhesion to a solid surface.

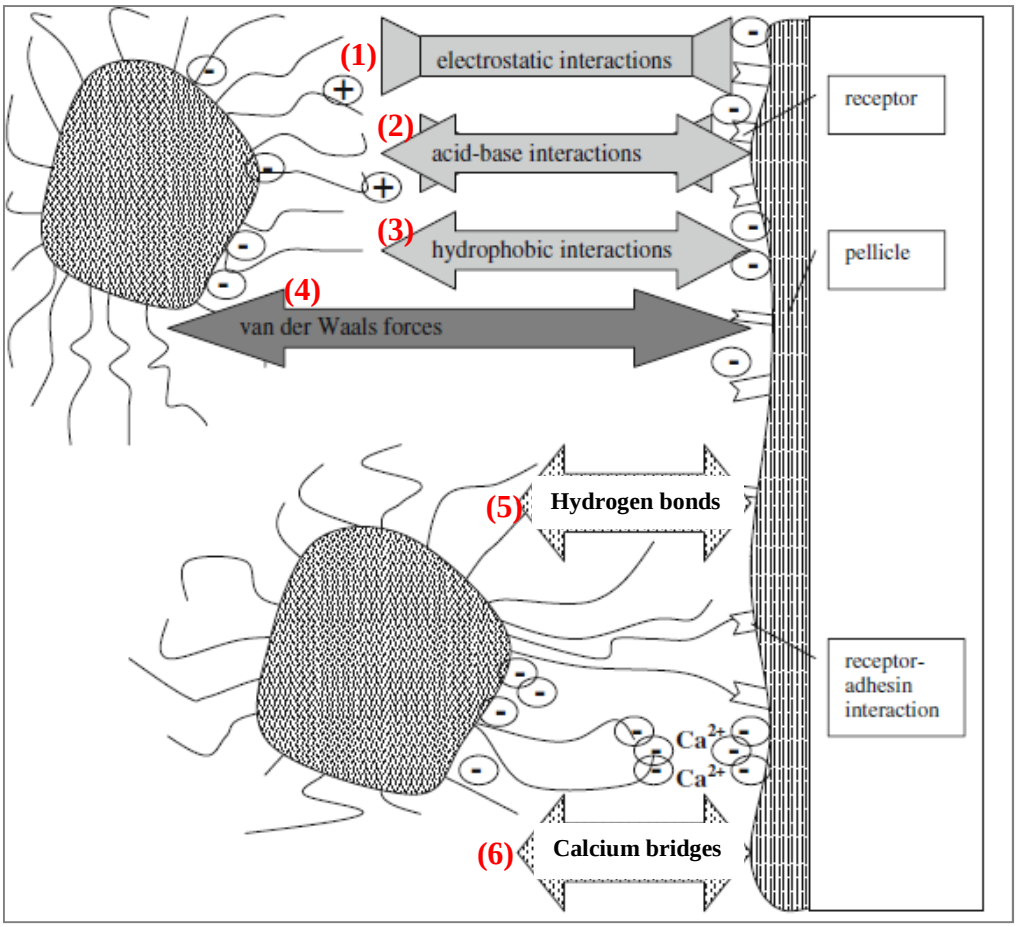


Figure 1.7 : Governing interactions of bacterial cell adhesion (Hannig & Hannig, 2009)

Table 1.7 : Summary of governing interactions in bacterial adhesion to solid surface

Figure7 Legend	Interaction	(Un) favorable + / -
(1)	Electrostatic	Repulsion
(2)	Acid-base	Dependence of cell surface molecules
(3)	Hydrophobicity	Attractive for hydrophobic cells and surface
(4)	Van der Waal forces	Always attractive, but acts when close enough
(5)	Hydrogen bonds	Cell surface molecules
(6)	Calcium bridges	Favorable to adhesion

I.5.4. Matrix of extracellular polymeric substances

The biofilm well developed also forms a “compact” and “gelatinous” structure where the bacteria are “stuck” in the “cement” of extracellular matrix. The extracellular matrix plays two major roles: i) in the cells adhesion to sand grain surface; ii) in the cells cohesion among themselves. The next section discusses this main structure of biofilm: extracellular matrix and more detailed: the organic fractions of extracellular matrix: extracellular polymeric substances (EPS).

I.5.4.1. Spatial organizations of extracellular matrix

The EPS can bond tightly around the outer space of bacterial cells, as capsules-like closely associated. The bacteria also produce slime-like EPS loosely bond with cells and soluble EPS which do not associate directly to the cells (Wingender *et al.*, 1999). Besides the organic fractions, the minerals are also bound in the extracellular matrix. The Figure 1.8 demonstrates the organizations bound EPS and soluble EPS:

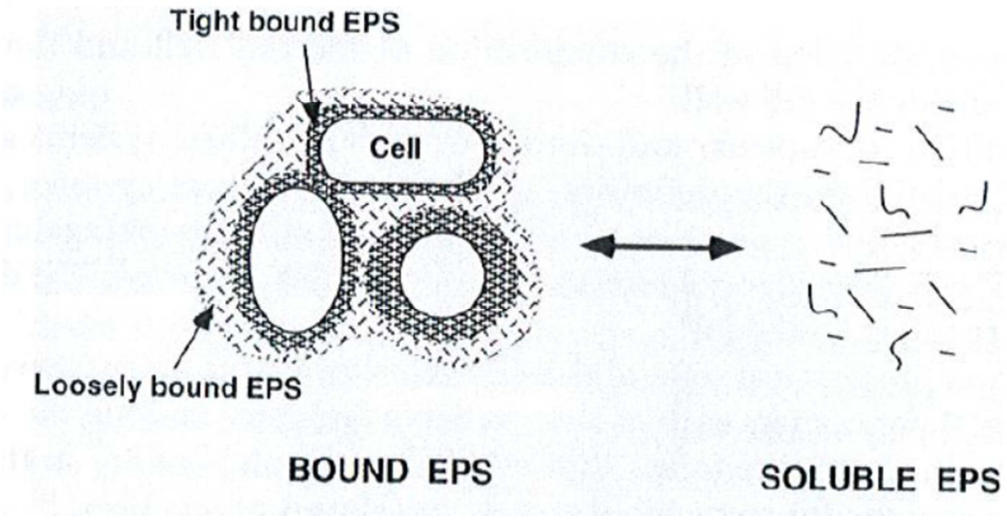


Figure 1.8 : Spatial organizations of EPS (Wingender *et al.*, 1999)

The way to separate two fractions is usually by centrifugation, where the polymers in the supernatant are soluble EPS and the polymers bound with the pellet are bound polymers:

- *Bound EPS*

The bound EPS can also be grouped into two subcategories: tight bound (TB) EPS and loosely bound (LB) EPS. The TB-EPS are closely associated with cellular envelops, and the LB-EPS are gel-like substances surrounding the cells, and can be considered as slime.

- *Soluble EPS*

The soluble EPS regroups the substances that are weakly associated and free molecule: including some slimes and soluble or colloidal macromolecules which however are not dissolved (Sardin *et al.*, 1991). Wingender *et al.*, (1999) indicated that the separation of soluble EPS from the matrix demands an extraction of weak energy (centrifugation…) while the bound EPS needs treatments allowing the dispersion of biomass (heat, reactive chemicals…).

I.5.4.2. Biochemical composition of EPS and mineral fractions

As mentioned, the matrix of EPS contains proteins, polysaccharides, nucleic acids, lipids as major biochemical components and also minerals…The biochemical fractions are changeable depends the nature of biomass, the type of substrates and other environmental factors.

❖ *Mineral fractions*

The mineral composition (cations, anions) plays an important role in the metabolism of bacterial cells. Mineral fractions present in the matrix in the different forms: ionized or particulates. The particulate mineral solids are formed by precipitation, such as Ca salts ($CaCO_3$, $Ca_5(PO4)_3(OH)$) and Fe salts ($FeO(OH)$, $FePO_4\ 2H_2O$) (Juang *et al.*, 2010). The multivalent cations (Mg, Ca, Fe…) create the ionic interactions with the ionized functional groups of EPS. The cations in the matrix are mobile and interchangeable (Higgins & Novak, 1997).

❖ *Proteins*

Proteins have an amazing range of structural and catabolic properties as a result of their various amino acid compositions. The proteins are amino acids chains linked by peptide bonds of various lengths. The side chains of α carbon atom of amino acids possess various functional groups (electrically charged, polar, or nonpolar) bring in various interactions like ionic, hydrogenous, or hydrophobic with other molecules (Riemer & Harremoës, 1978). The properties and functions of proteins are related to their spatial configurations which depends on the amino acids compositions (water solubility, colloids solution, denaturation, and spectrofluorimetric properties…). The mechanisms of protein secretion are the synthesis takes plan in the cytoplasm and the molecules are then excreted into the environment (Rodgers *et al.*, 2004). Proteins can also associate with polysaccharides (glycoprotein) and lipids (lipoprotein) (Bourven *et al.,* 2012). Okubo & Matsumoto, (1979) utilizing the combination of size exclusion and infrared microscopy on EPS from activated sludge, found that except high molecular weight proteins (from 45 to 670kDa), it existed also a group of small size proteins (<1kDa) in smaller amount that were associated with polysaccharides. Similarly, the presence of lipoproteins as one subfamily of proteins was also proven by the same technique (Kristiansen, 1981c). The proteins represent a group of molecules possessing various

functions: i) metabolism with the enzymes (Frølund *et al.*, 1995); ii) structural: The structural function is first due to the diversity of functional group of amino acid which can interact with other molecules/mineral element and second with specific structure (in gram negative bacteria), the lectins-like proteins which link sugar group (Higgins & Novak, 1997).

- *Polysaccharides*

The polysaccharides are the biopolymers the most studies since they represent the majority of EPS matrix components in pure bacteria cultures and it is also the reason that some studies referred the exopolysaccharides as the EPS (Costerton *et al.*,1995). Most microorganisms secrete polysaccharides. The majority of these compounds are neutral sugars (glucose, galactose, and mannose) (Dignac *et al.*, 1998). Polysaccharides are ubiquitous biopolymers build up by monosaccharide of variable molecular weight. Most of them are hydrophilic with the presence of hydrogen bond. The synthesis of the polysaccharides can be intracellular or extracellular (Kumar *et al.* 2007). The polysaccharides in EPS matrix are either in forms of capsule covalently associated with cellular membrane (lipopolysaccharides) or in forms of slime weakly joined with the cells (Kumar *et al.*,2007). Certain polysaccharides also contain variable groups such as: hydroxyl, acetyl, phosphate. Some of them are capable to bond with Ca^{2+} ions, for example, bacterial alginates which represent a few exopolysaccharides. The alginates have certain capacity to form a gel and this capacity depends on the presence of mannuronic and guluronic acids. Many alginates possess the sequence of poly-$_L$-guluronic acid which can bind Ca^{2+} very effectively within the “egg-box” structure where the distribution of Ca^{2+} in the gel were most observed on the surface (Geddie & Sutherland, 1994; Lin *et al*, 2010).

- *Humic substances*

The reference of humic substances as the EPS compounds is abusive. By definition, the humic substances come from the soil, where they can be resulted from long evolution process. Thus researchers refer these compounds as humic-like substances and this group of molecules is very heterogeneous. They come from the biodegradation or chemical degradation of environment organic residues (Franciesco *et al*, 2002). Their main components are phenol, amino acids, and sugars of small sizes (Wuertz *et al*, 2001). They contain functional groups such as: alcohol, phenol, carboxylic, lactones…

❖ *Nucleic acids*

DNA and RNA are two groups of polymers of nucleotide chains. One nucleotide contains a sugar of five carbon atoms (deoxyribose for DNA and ribose for RNA), a phosphate group, and an amino group. The nucleic acids represent only a small amount in EPS matrix extracted from wastewater biomass (Frølund *et al,* 1996). At first, the extracellular nucleic acids were considered as a product and an indicator of cellular lyses. Later, researchers found that many microorganisms secrete large amount of extracellular nucleic acids (Steinberger & Holden 2005). In multi-species biofilm, high content nucleic acids were detected at the outer layer of micro-colonies (Allesen-Holm *et al.*, 2006) which is in accordance with their roles in the communication among the cells.

I.5.4.3. Influencing factors of EPS secretion

The EPS play a role of tampon as protection and nutrients for the microorganisms in the matrix, for example, the soluble EPS are substances easy to metabolize to be the carbon and energy source (Laspidou & Rittmann, 2002). Some environmental factors affect the secretion of EPS:

❖ *Stress*

The presence of the stress factors simulates the EPS synthesis: low substrate content (Hoa *et al.,* 2003); salinity (Mishra & Jha, 2009); presence of metal ions (Mikes *et al.,* 2005). An increase in soluble EPS was observed at low substrate condition (Aquino & Stuckey, 2004); Avella *et al,* (2010a) have observed an increase of soluble EPS and that the protein fraction increased during the presence of antibiotic in activated sludge biofilm; A higher amount of polysaccharides has been observed in some resistant bacterial strains pure culture with the presence of metal elements (Kazy *et al.,* 2002).

❖ *Physical conditions*

The shear forces influent the composition of EPS matrix. The EPS quantity of sludge increased under higher shears and higher aeration conditions (Adav *et al.,* 2008a). The soluble EPS increase by the release from the aggregates took place under enhanced flow hydrodynamics and shears (Aquino & Stuckey, 2004). The increase in sugar fraction in EPS

were shown under higher aeration condition (Shin *et al*, 2001), and the loss in EPS were observed under anaerobic sludge (Nielson *et al*, 1996).

❖ *Characteristics of substrates*

The ratio of nitrogen/carbon in substrates seems affects the productions of EPS, however this effect of the nitrogen ration is not quite clear and accordant in studies. Some authors claimed higher productions both in soluble and bound EPS while the increase of the N/C ratio (caseins/starch from 2 to 8) (Arabi & Nakhla, 2008), and some authors found that the increase in N/C ratio only induced the increase in weakly bound EPS production (Ye *et al*, 2011).

I.5.4.4. Functions of EPS

As mentioned in the “bacterial adhesion”, one of the functions of EPS is to facilitate the irreversible adhesion and to initiate the colonization. Besides these fundamental functions, the EPS also benefit the stabilization of biofilm by involving in different interactions in the biofilm, especially in the biofilms of wastewater treatment. These particular functions will be developed after a summary of EPS functions.

❖ *General functions of EPS as biofilm*

Some other general functions have been attributed to EPS which are summarized in Table 1.8:

Table 1.8 : Summary of functions of EPS (Wingender *et al.*, 1999)

Function of EPS	Relevance	Reference
Aggregation of the cells and formation of biofilms	Bridging between cells and inorganic particles, immobilization of bacterial populations	Ellis & Aydin, (1995); Villermaux & Van Swaaij, (1969)
Protective barrier	Protection of bacteria against biotic and abiotic noxious influences from the environment	Riemer *et al.*, (1980)
Retention and sorption	Prevention of desiccation of water, sorption , digestion, accumulation of nutrients and inorganic ions from environment	Stevens *et al.*, (1986)
Structural element of biofilms	Mediation of mechanical stability of biofilms through conjunction with covalent cations or between neighbor EPS; determination of shape of EPS structure	Riemer & Harremoës, (1978)

❖ *Roles of EPS in biofilm structural stability*

The stability of biofilm refers to the capacity of the aggregates to resist to the hydrodynamic constrains and to the shear forces (Sheng *et al.*, 2006b).The EPS play an important role in the stabilization by interacting between cells or with multivalent cations and also by the hydrophobicity…

- The proteins and sugars contribute to the stability of biofilm which are confirmed by the deflocculation after the hydrolysis of proteins and polysaccharides by adding the enzymes (Cammarota & Sant'Anna., 1998; Yang *et al.*, 2004).

- The production of EPS also neutralizes the negative charge of cell surface and reduces the repulsive electrostatic force and the cell adhesion to the aggregates can be benefited by the polymeric interactions (Tsuneda *et al.*, 2003a).
- The hydrophobicity helps the cells attach to aggregates and allows them to stick and keep the stability of the aggregates (Zita & Hermansson, 1997). The hydrophobicity is associated with the presence of fibrillar structures and specific proteins, but not with the presence of polysaccharides (hydrophilic) (McNab *et al.*, 1999; Singleton *et al.*, 2001; Arabi & Nakhla, 2008b).
- The lectin-like proteins located in the appendices of cells interact with the bacterial polysaccharides by the hydrogen link (Mirelman, 1986; Bush *et al.*, 1999).
- The presence of divalent cations also reduce the repulsive force between cells and favorites the aggregation. The non-monovalent cations also intervene the stabilization of EPS molecules by ionic interactions (Higgins & Novak, 1997).

❖ *Role of EPS in the sand filtration process*

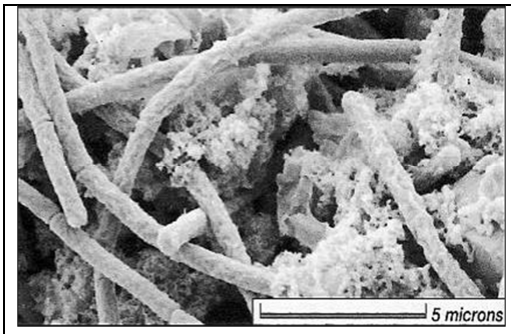
The EPS are considered as the main components of biofilm which constitute the biological clogging. Studies showed that the presence of EPS at mature state reduces the infiltration rate (Rodgers *et al.*, 2004a; Schijven & Hassanizadeh, 2000). But the spatial distribution of EPS around sand grains also ammeliorates the repartition of substrates and increases the residence time which benefits the purification process of sand filtration. However the EPS evolution during operating time and as function of filter depth is rarely documented and few studies have been carried out on the their more detailled characteristics such as their compositions or molecular sizes.

I.6. Characterization- monitoring of biofilm (cells + EPS)

The complex nature of biofilm makes the investigations difficult. The biofilm is investigated by various techniques from different aspects. The most of these techniques are destructive to the biofilm. General information can be provided by simple measurements, such as: totality of biofilm (amount as organic matters, or as Carbon or Nitrogen), thickness and structures of biofilm; more detailed information on the cells and EPS may need several

steps of treatments and followed by specific analytical techniques. An overview of several categories of biofilm investigation techniques is presented in Table 1.9:

Table 1. 9 : Biofilm investigation techniques overview

<table>
<tr>
<td> 
</td>
<td>Biodiversity: microbial genomic level characterization of the cells in biofilm (molecular techniques):
❖ PCR based molecular techniques: DGGE, PFGE, RISA and Pyrosequencing™…
❖ Non-PCR based molecular techniques: FISH, GFP…</td>
</tr>
<tr>
<td rowspan="2">Totality, surface and interface of biofilm characterizing techniques:
❖ Total amount (VDW, C or N contents of biofilm)
❖ Visualization of biofilm by microscopic techniques (SEM, CLSM, STXM…)
❖ Scattering (spectroscopy) techniques (ATR-IR, NMR, X-ray…)
❖ Nondestructive techniques: Microsensors (electrochemical, fiber-optic microsensors)</td>
<td>EPS matrix: biochemical level characterization of the extracellular fractions in biofilm (separation techniques+ analytical techniques):
❖ EPS extraction (physical, chemical or combined methods)
❖ Molecular separation (GC, LC, SEC, FFF…)
❖ Quantitation of biochemical fractions (spectrophotometry)
❖ Analytical detection (MS, atomic spectrometry, electrochemical, optical detectors)</td>
</tr>
<tr>
<td>Microbial activities: physiological level characterization of the cells in biofilm (specific compounds detections):
❖ Enzymatic activities (ATP, hydrolysis, dehydrogenase…)
❖ Cells extraction – culture – count…</td>
</tr>
</table>

I.6.1. Biofilm observation (microscopic techniques)

Microscopic techniques have high potential in analysis of biofilms, but natural biofilm can develop into thick package that may limit light penetration into the biofilm matrix hindering the use of optical techniques, such as the light microscopy, which is advantaged with its simplicity and rapidity; however also limited by its low resolution (Lazarova & Manem, 1995). The development of Confocal Laser Scanning Microscopy (CLSM) and epifluorescence microscopy has extended the possibilities of in-depth visual 3-dimensional

(3-D) observation of biofilm structure and provided a bridge between light microscopy and electron microscopy (Zhang & Fang, 2001). The carbohydrate of *Pseudomonas aeruginosa* biofilm has been visualized and characterized by using fluorescently labeled lectins in combination with a epifluorescence microscope and a CLSM (Strathmann *et al.*, 2002). The Scanning Electron Microscopy (SEM) provides high resolution images that allows the visualization of the biofilm microstructure including the visualization of EPS and cells (Eighmy *et al.*, 1983). The Scanning Transmission X-ray Microscopy (STXM) shares similar principal with SEM. A multi – microscopy combination (CLSM-STXM-SEM) has demonstrated the distribution of macromolecules (protein, polysaccharides, lipid, and nucleic acids) of the biofilm in river sediments (Lawrence & Neu, 2003). STXM is also capable of detecting the absorbed metals in biofilm.

I.6.2. Characterization of the population of biofilm – Cells

The cells are considered as the biomass which is responsible of the wastewater purification process for both suspended and fixed biomass reactors. It has always been the subjects in water treatment research the total content, the biodiversity and the physiology of the cells, otherwise the biomass in the biofilm.

I.6.2.1. Total content assessment of biomass

- *Volatile Dry Weight (VDW): Total organic matters*

The VDW (weight loss between 105 and 550°C) is often used to estimate the total amount of biomass and this method is also generally used to assess the total organic matters of soil samples. The biomass may be overestimated by heating weight loss, since the structural water of clay minerals also loss weight during heating along with the organic materials (Rice, 1974).

- *Biomass carbon and nitrogen determination*

The biomass carbon and nitrogen assessments are generally applied to fixed biofilm samples, especially often carried out to quantify the living microbial biomass in soil or in sediments. The techniques are referred to fumigation – extraction (incubation) methods or substrate – induced respiration method (Wardle, 1992).

❖ *Revivifiable cells count– Extraction and enumeration of cultivable microbes*

Quantitative and representative recovery of microorganisms from environmental samples is essential in understanding the ecosystem function. The microbial enumeration is a screening-level tool which can be used to evaluate the response of soil microorganisms or attached bacteria in artificial porous media, and the geometric distribution of microbial populations and communities. The enumeration of microbes in soil or other filter media requires the sample preparations which generally involves the dispersion of substratum sample and the extraction of attached cells from substratum which involves a variety of binding mechanisms (Trevors & van Elsas, 1995). Because of the strong binding between cells and substratum, the severe cell damage may be a result of breaking these binding. The ultrasonic treatment and low speed centrifuge as dispersion and extraction can only recover a fraction of dislodged cells (Lindahl & Bakken, 1995). The enumeration of cells is based on the revivifiables aerobic flora which includes aerobic and facultative anaerobic bacteria. In soil, population densities of TRHs within background soils usually range between 10^4 and 10^7 CFU/g soil (Maila *et al.*, 2005). The study of Chabaud (2007) showed that the number of cultivable cells recovered from a sand filter were relatively stable during the operating period.

I.6.2.2. Biodiversity – Molecular techniques

Genetic information is often required in microbial biodiversity and taxonomy investigations to explore the molecular basis of biofilm formation. A reconstruction of near compete genomes or genome sequences of microorganisms is possible using genomic datasets in combination with molecular techniques. These techniques include polymerase chain reaction (PCR) for DNA amplification, followed by pulse field gel electrophoresis (PFGE), denaturing-gradient gel electrophoresis (DGGE) and more detailed information can be obtained by coupling with other analytical methods like mass spectrometry and microscopic techniques (Aoi, 2002; Meays *et al.*, 2004). There are also molecular fingerprint techniques that do not involve PCR amplification, such as fluorescence in situ hybridization (FISH), by applying ribosomal RNA-targeted oligonucleotide probes could be visualized with the help of fluorescence quenching, or technique such as ribosomal intragenic spacer analysis (RISA) based on PCR amplification products. RISA has been employed to evaluate the evolution of bacterial communities' complexity in sand filtration columns and demonstrated an increase of bacterial communities throughout the operation time (0-138 days) (Chabaud, 2007). FISH has been successfully employed to explore bacterial communities in activated sludge, marine,

freshwater environments, marine sediments, soil (Wagner *et al.*, 1993; Amann *et al.*, 2001). FISH conjunction with CLSM has allowed defining the 3-dimentional distribution of microbial populations in mixed species biofilm of different environments (Moller *et al.*, 1996; Manz *et al.*, 1999).

I.6.2.3. Physiology of cells – Enzymatic activities

The enzymatic activities can be associated with active cells, cell debris as well as being complexed with minerals or colloids (Burns, 1982). Various techniques of quantifying microbial activities by enzymatic measurements exist in the literature. While the activity of many extracellular hydrolases is probably a result of enzymes associated with some or all these compounds, dehydrogenase assays measures intracellular catalysis and are more likely to be correlated with the activity of extant cells (Dick, 1997). The fluorescein diacetate (FDA) hydrolysis is used as a general indicator of soil hydrolytic activity. The determination of FDA hydrolysis is simple, rapid and sensitive, except when activities are low, it requires long time of incubation (Okubo & Matsumoto, 1983). The adenosine triphosphate ATP content can be associated with the soil biomass, however the ATP content represents only a small portion which is active (Beach *et al.*, 2005).

I.6.3. Characterization of the EPS (separation + quantification + analysis (monitoring))

The matrix of EPS forms a shelter to protecting the cells from environmental stress and providing suitable conditions for proliferation. Studying the EPS requires the separation from the biofilm sample first, which is commonly known as the extraction, then further investigation can be done by analytical techniques. As the extraction of EPS is destructive to biofilm samples, the following-up of EPS evolution throughout time is quite unlikely.

I.6.3.1. EPS Extraction methods

Either soluble EPS or bound EPS need to be separated from the matrix or the biomass to carry out further investigations, and certain EPS components are divided purified from the extracts for more detailed analysis. The main difficulty in EPS extraction procedure is to obtain high extraction efficiency without unwanted cell lyses and disruption of

macromolecules. The main forces involved in binding EPS are Van der Waal force, electrostatics interactions mediated or not with multivalent cations, hydrogen bond, and hydrophobic interactions. The main dominating force varies from biofilm to another, thus the extraction method should be chosen according to each case, and no universal extraction method exists for a quantitative extraction of bound EPS compounds from microorganisms growing in suspensions or in aggregates or attached surfaces (Sardin *et al.*, 1991). The EPS yields depend on extraction time, shear force and the dependences are different for various EPS compounds. The extraction methods are classified into 2 categories by their shears force they provide:

- *Physical methods:* a shear applied to extract EPS by shaking, mixing, centrifugation, and sonication or heat…

- *Chemical methods:* treatments include addition of various chemicals that can break the linkage in the EPS matrix, such as: alkaline treatment with the addition of NaOH, complexing agent like ethylenediaminetetraacetic acid (EDTA), cation exchange resin (CER) (this method was actually a mix between physical (shaking with resin) and chemical (exchange with Na^+ and multivalent cations from the biofilm) extractions)…

The combination of physical-chemical extraction is more and more applied. Furthermore, the biofilms in wastewater treatment systems are highly heterogeneous. The matrix of EPS contains water, proteins, polysaccharides, nucleic acids, lipids and also bound minerals…Their biochemical fractions depends the nature of biomass, the extraction and analysis techniques, and also the type of substrates. Many studies have been carried out with wastewater EPS by comparing different extraction procedures and the advantages and shortcoming of some of these methods are summarized in the Table 1.10:

Table 1.10 : Studies on EPS biochemical composition of wastewater biofilms

Extraction method	**Experimental conditions**	**Advantages**	**Shortcomings**	**References**
Centrifugation (control)	low speed: 5000-10000g	Extraction of LB EPS and soluble EPS; less cell lyses	Not effective enough for extraction of TB EPS	Liu & Fang, (2002)
Ultrasound	Probe: 37W output for 15-420s; Bath: 20kHz, 120W input, 3×2min	Fair extraction efficiencies for TB-EPS, no disruption for further investigation	Cause proteins, nucleic acids leakage and cell lyses	Jorand *et al.*, (1995) Liang *et al.*, (2010)
Heat	80°C, 1h	High extractions efficiencies compared to other physical methods	Cause cell lyses and disruption of macromolecules (ex: denaturation of proteins)	Frølund *et al.*, (1996)
CER	250g or 650g DOWEX/l, 300 or 600rpm, from 1 to 8h	Higher efficiencies for proteins compared other physical methods	Less compatible with porous media extraction	Frølund *et al.*, (1996) Dignac *et al.*, (1998)
EDTA	2% for 3h at 4°C	Higher extraction yields than physical methods	Cause higher cell lyses than physical methods Disruption of macromolecules; contamination	Comte *et al.*, (2007)
Formaldehyde + NaOH	0.4V of NaOH (1M)/1V agitation for 3h at 4°C	Good extraction efficiencies; less cell lyses than other chemical methods	Contamination of chemical agents; disruption of macromolecules by additions of NaOH	Liu & Fang, (2002)

The choice of extraction method applied in each case remains a compromise between the high yields of biochemical components and the absence of contamination by chemical reagents or by intercellular materials for further investigations. The involving of chemical reagents and thermal treatments often interfere the chromatograms of size exclusion chromatography due to the denaturation and disruption of macromolecules, such as protein-like compounds (Bourven *et al.*, 2013). Physical methods such as ultrasound appear having modest recovery efficiencies but no interferences to any other analysis followed. In this case, the ultrasound was chosen over CER in cases of EPS extraction since the ultrasound has fairly equal extraction efficiencies for the major groups of EPS (Comte *et al.*, 2007). The ultrasound treatment: probe (Probe Bandelin GM 70, 60W during 90s, Labanowski, 2004) and bath (Sonicator, bath capacity of 2L, 40W-100W, Liu *et al*., 2009) are largely used for soil and activated sludge EPS extraction. The study of Yu et *al.* showed that ultrasound probe treatment extracted more types of extracellular enzymes than ultrasound bath treatment, but might also lead to cell lysis (Yu *et al*., 2009).

I.6.3.2. Physic-chemical properties of EPS and Characterization methods of EPS

❖ *Quantification of organic components with classical colorimetric methods*

The contents of the main EPS components are often assessed via the colorimetric methods. The chemicals interact with the certain functional groups which are specific for target components, and the intensity of generated color is measured by UV/Visible spectrophotometer. The result relies on a known reference which establishes the calibration curve with a series of known concentrations. Several methods exist for each component in order to improve the reliability. Some common used methods are summarized in the Table 1.11.

The EPS resulted from the biomass of wastewater purification is highly heterogeneous and this great diversity reduces the efficiencies of colorimetric assays. For example, the different colorimetric assays for proteins present their own advantages and inconveniences: the protein contents determined by Lowry method are interfered with humic-like substance and thus overestimated. Lowry method has been modified in order to take account the humic-like compounds (Frølund *et al.*, 1996). On contrary, this modification tends to underestimate the protein contents by taking account humic-like compounds even when they are absent (Ras *et*

al., 2008; Avella *et al.*, 2010). The Bradford method does not interfere with other organic compounds but also underestimates the protein contents due to different colorimetric reactions according to the type of proteins (Raunkjær *et al.*, 1994). According to Ras *et al.*, (2008) and Raunkjær *et al.*, (1994), the BCA method also has different reactions with the types of proteins and overestimates the proteins by taking account some polysaccharides.

Table 1.11 : Colorimetric assays of certain EPS components

Component	Method (References)	Reagent	Target functional group	Standard	Calibration sensibility
Proteins	Lowry (Lowry *et al.*, 1951)	Folin-Ciocalteu, 5% $CuSO_4$	Peptide links (>2) and phenols	Bovineserumalbumin (BSA)	0.04-0.2 g/L
	Bradford (Bradford, 1976)	CoomassieBrilliant Blue	Peptide links (>8) rich in arginine	BSA	0.04-0.2g/L
	BCA (Smith *et al.*, 1985)	Bicinchoninic acid (BCA), $CuSO_4$	Protein-BCA-Cu^{+1}complexion: biuret reaction	BSA	0,2-1 g/L
Humic-like substance	Modified Lowry method (Frølund *et al.*, 1996)	Folin-Ciocalteu	Phenols	Humic acid	0.04-0.2 g/L
Polysaccharides	Dubois (Dubois *et al.*, 1956)	5% Phenol	Monosaccharide with>5 C(H_2O)	Glucose	0.02-0.1 g/L
Nucleic acids	Burton (Burton, 1956)	0.6% Diphenylamine	2-desoxyribose	Calf thymus DNA	0.005-0.05 g/L

❖ *Molecular weight and HPSEC*

EPS are composed of molecules of variable molecular weight (MW). The determination of the apparent MW (aMW) distribution is achieved by high-pressure size exclusion chromatography (HPSEC). This method provides a separation of crude EPS extracts based partly on molecular hydrodynamic size and partly based on non-size exclusion separation

mechanisms. The signal is generally detected by UV absorbance or rafractiometric detection (Frølund *et al.*, 1996). The infrared spectroscopy is sometimes coupled with SEC to identify the chemical nature. It has been successfully applied to characterize wastewater sludge exopolymers (Frølund *et al.*, 1996; Görner *et al.*, 2003; Garnier *et al.*, 2005; Comte *et al.*, 2007; Simon *et al.*, 2009), and the HPSEC fingerprints were generally recorded at the wavelength of 210nm and 280nm, detected by UV absorbance: these authors suggested that proteins have relatively large apparent MW ranged from 10 to 670kDa, and that polysaccharides have low aMW smaller than 1kDa. Frølund *et al.* (1996) found similar profiles of HPSEC fingerprints from the EPS of different origins. Comte *et al,* (2007) demonstrated that HPSEC fingerprints differed with the chemical extraction whereas the control and physical extractions exhibited similar fingerprints. Higher aMW (up to 1200kDa) compounds presented in strongly bond EPS or capsular EPS and the majority was proteins and nucleic acids. Whereas in weakly bond EPS or slime EPS, the aMW ranged from 0.3 to 20kDa (Andersson *et al.*, 2009; Yu *et al.*, 2009). With the combination of SEC-fluorescence detection, the protein-like fingerprints have been specified by the chosen Ex/Em wavelength and exhibited heterogeneous MW ranged broadly from large sizes of >600kDa to small sizes of <9kDa (Bourven *et al.*, 2012). Later, humic-like compounds fingerprints were also specified by fluorescence detector, the main aMW of humic-like substances of both activated sludge and anaerobic granular sludge ranged in low MW region: from 1.4 to 6kDa (Bhatia *et al.*, 2013).

- *Surface properties: Hydrophobicity and surface charge*

Both hydrophobicity and surface charge belongs to the surface properties of EPS and these two characteristics are often associated in the studies. The hydrophobicity is one of the fundamental interactions in bacterial aggregates and the hydrophobic interactions have great influence on the settleablity, dewaterability and bioflocculation of sludge. The hydrophobicity of EPS is characterized by XAD-8 and XAD-4 resin which divide soluble EPS (at pH=2) into three fractions filtering through XAD resins: 1) fraction sorbed onto the XAD-8 resin: hydrophobic substances; 2) fraction sorbed onto the XAD-4 resin: hydrophilic substances and 3) unsorbed fraction: non-sorbed hydrophilic substances (Martin-Mousset *et al.*, 1997). Several techniques can estimate the cell surface charge: the determination of zeta potential, the acid-base titration and the colloids titration (Jorand *et al.*, 1998). The soluble EPS at pH=2 showed a significant protein fraction and that no carbohydrates have been found in the

hydrophobic fraction which indicated that the carbohydrates do not involve in the hydrophobic interactions (Jorand *et al.*, 1998; Arabi & Nakhla, 2008a). The surface charge is related to the ionizable groups presented on the sludge surface, and also strongly depends on the composition of EPS matrix. Studies indicated an inverse correlation between the surface charge and hydrophobicity, both are strongly influenced the proportion of EPS components, especially increased with the increment of the ratio proteins/carbohydrates (Liao *et al.*, 2001; Mikkelsen & Keiding, 2002; Sponza, 2002; Wang *et al.*, 2006). Extracellular proteins contribute the hydrophobic properties of EPS due to their high proportions of hydrophobic amino acids alanine, leucine and glycine (Higgins & Novak, 1997). The study of Wang et *al.*, (2006) showed that the negative surface charge exhibited a positive correlation with the increase of total EPS content; however the EPS presented hydrophobic property due to the increased ration PN/PS, which indicated with abundant EPS, the attractive force was dominated by polymeric interactions in cell adhesion.

- *Identification of EPS components*

There are analytic tools permitting the identification of certain molecules of classic EPS components after an appropriate separation and purification. Attempts have been made to identify extracellular protein of activated sludge: Park *et al.,* (2008) submitted 11 intense bands of polypeptides resulted from Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to liquid chromatography coupled with mass spectroscopy (LC-MS/MS) and the positive hits indicated that the diverse origins of protein in EPS matrix, besides the presence of the extracellular proteins resulted from bacterial defense, cell surface outer membrane and cell appendage, proteases from influent remained in the EPS matrix. 16 amino acids have been separated with the help of high performance liquid chromatography (HPLC) and detected by spectrofluorimetric detector and the results also showed that several possibilities of proteins source in EPS matrix including bacterial excretion and influent residues (Dignac *et al.*, 1998). Gas chromatography (GC) coupled with mass spectroscopy or flame ionization detector has been often applied to identify the monosaccharaides and lipids presented in the extracts according to their different retention time (Dignac *et al.*, 1998; Gloaguen *et al.*, 2004; Andersson *et al.*, 2009).

❖ *General view of protein-like and HS-like with Excitation/Emission Matrix*

The fluorescence spectroscopy has been employed to characterize the fluorophores contents in natural organic matters. The protein-like and humic-like compounds are two great categories of the fluorophores in EPS matrix. Several studies have applied the 3D fluorescence excitation-emission matrix (3D-EEM) to characterize the presence of proteins and humic substances in EPS extract of activated sludge: the spectra obtained are generally composed of 3 main peaks: 2 peaks of emitted fluorescence of protein-likes compounds and 1peak of humic-like substances (Esparza-Soto & Westerhoff, 2001; Sheng & Yu, 2006; Domínguez *et al.*, 2010; Bhatia *et al.*, 2013). This technique has been considered as a rapid tool of qualitative analysis of EPS extracts in sludge.

I.7. Conclusion of literature review

From the information given by the previous studies, the essential characteristics of the technique, the conception and the functioning of OWTS systems, especially the sand filters have been explained. The majority of these studies allow identifying the operating parameter (loading charge, dosing frequency, material natures…) which influence greatly the purification performance and the duration of the installations. The milieu of sand filter receiving septic effluent is an environment very complex and heterogeneous, and the complexity and the nature of the interactions among physical, chemical and biological process taking place in this unsaturated environment makes it difficult to renew the knowledge. It appears that the biofilm develops mainly at the surface layer of the filter and participates importantly the reduction of different pollutant. At the same time, it provokes the phenomena of clogging which may interfere the functioning of the filter. Furthermore, the influence of the filter material is still quite unclear and the lack of natural deposit of alluvial sands in certain areas simulates the researches of similar materials, such as crushed sands. In this study, the interests were brought to the characteristics of crushed sands (materials, hydrology, purification and biomass distribution evolution and microbial activities) and the influence of these characteristics on the possibility of using them as filter media for domestic wastewater treatment (purification efficiencies; biological clogging caused by biofilm).

The strategy of experimental study:

With these interests, this study is based on the comparative characterization between selected alluvial and crushed sands. The experimental study was carried out around following points (Table 1.12):

Table 1.12 : Strategy of experimental study

<table>
<tr><td colspan="3">Comparative study of crushed sands and alluvial sands</td></tr>
<tr><td>1st phase</td><td>2nd phase</td><td>3rd phase</td></tr>
<tr><td rowspan="2">Conception and construction of sand filtration columns system</td><td rowspan="2">Experimental study of material characteristics as foundation of comparison</td><td>Monitoring study of purification efficiencies of main pollutants by two types of sands and at different depths</td></tr>
<tr><td>Monitoring study of biofilm components distribution in the profile of filter depth and their evolutions throughout operating period: biomass quantification; evolution of the size of macromolecules; microbial activities…</td></tr>
</table>

The monitoring of the abatements of main pollutants was referred to the classic physico-chemical analysis of inlet and outlet effluents. The analyzed parameters represented the organic pollutants, nitrogen and phosphorous pollutants.

The system allowed the sampling in depths inside the sand columns. The monitoring of biomass (totality) and their components (cells and extracellular matrix respectively) was based on the combination of simple rapid methods and more pushed forward analysis: the choice of methods was based on the purpose, quantity and quality of samples. Due to the nature of comparative study, identical analysis was applied to all the sands.

Part II: Experimental study and result discussions

Le sable de rivière est couramment utilisé comme matériaux de remplissage pour les biofiltres utilisés en système d'assainissement autonome selon les recommandations de la Norme Française (DTU 64.1). Les réacteurs de filtration biologique garnis de sable ont été largement étudiés en termes de procédé et de comportement hydrodynamique. Ce système avec comme matériau un sable de rivière, est considéré comme stable et son efficacité épuratoire parfaitement décrite et maitrisée (Wanko *et al.*, 2005). Le changement de matériaux de garnissage peut modifier les qualités attribuées au procédé en considérant que peu d'études ont été réalisées, en particulier sur les agrégats concassés. L'objectif de ce travail est de regarder l'impact de la nature du garnissage des filtres biologiques sur:

- le fonctionnement épuratoire du procédé
- le développement de la biomasse et une partie spécifique de celle-ci : les EPS

Dans ce sens, 3 chapitres chacun comportant les résultats et la méthodologie adoptée sont présentés:

Le premier chapitre sera consacré à la caractérisation des matériaux de remplissage du réacteur et leurs conséquences directes sur le comportement du réacteur: hydrauliques et hydrodynamique.

Le second chapitre sera consacré à l'observation des effets éventuels des matériaux de garnissage sur le fonctionnement épuratoire du filtre. L'étude s'est focalisée sur la mise en place du procédé jusqu'à l'obtention d'un état stationnaire apparent soit sur une période de 360 jours.

Enfin dans le dernier chapitre nous nous intéresserons au comportement de la biomasse développée dans le réacteur ainsi qu'aux EPS en fonction des matériaux mis en œuvre. Cette approche est réalisée en suivant l'évolution de la matière organique du biofilm pendant le procédé.

Pour cette étude, le pilote mis en place est constitué d'un ensemble de réacteurs de 70cm de profondeur qui respecte la recommandation propre à l'assainissement individuelle autonome (DTU 64.1).

Chapter 1: Study of filter materials and filtration reactors

Introduction:

Quatre matériaux de remplissage seront étudiés : deux sont des sables de rivières (RS1 et RS2) et deux sont des agrégats concassés (CA1 et CA2). RS1 est prévu pour être « le sable type » pour un fonctionnement optimal car répondant à une répartition et à une taille moyenne reconnue pour son efficacité (Cemagref, Liénard *et al.,* 2001).

En effet, la répartition des tailles des matériaux de remplissage des biofiltres est un paramètre majeur et classiquement pris en compte dans le choix des matériaux (NF DTU 64.1).

Le choix des différents matériaux a donc été guidé par ce paramètre dont les limites pour un procédé de filtration en assainissement autonome sont définies par la norme NF DTU 60.1. A côté de ce paramètre de distribution des tailles, d'autres paramètres de caractérisation physiques (masse volumique apparente et réelle, porosité, surface spécifique) et chimique (composition minérale et comparaison des cations relargués) ont été mis en place.

En effet, les caractéristiques physiques influencent l'hydrodynamique des réacteurs alors que les caractéristiques chimiques et morphologiques influenceront directement la mise en place du biofilm (adhésion et formation). L'excès de cations monovalents par exemple induit une désagrégation du biofilm (Higgins & Novak, 1997). L'hydrodynamique et l'hydraulique influenceront d'une part les échanges de substrats dont le plutôt la distribution des apports en substrats (matière organique, eau, O_2) au biofilm (Liu *et al.,* 2004), mais également le développement du biofilm par des effets mécaniques plus ou moins prononcés dans le réacteur (zones de cisaillement ou zones d'échange).

Les conséquences directes (les efficacités du traitement) sur le réacteur seront évaluées dans ce chapitre à l'aide d'un suivi classique des paramètres physico-chimiques.

1.1. Characterization of packing materials (experimental procedures of packing material characterization

1.1.1. Packing materials

The samples have been collected from various quarries in France. The alluvial sands originate from the river Loire. The crushed aggregates are originated from sandstones and sandstones are mixture of mineral grains and rock fragments. Among these samples, not all the aggregates fitted the required granulometric zone, even though they were indicated by quarries as for filter usage. 2 river sands and 2 crushed aggregates have been chosen to pack the columns. During the filling, the materials were compressed by water in order to avoid the compression during the operation, and this step also washed the aggregates by eliminate the dusts. The natures and quarries of chosen aggregates are summarized in Table 2.1.1:

Table 2.1.1: Quarries and natures of materials

Filter materials	RS1	RS2	CA1	CA2
Quarry name	Vritz	Les Alleuds	Mouen	Vaubadon
Nature	Alluvial (Loire)	Alluvial (Loire)	Crushed (Feldspathic sandstone)	Crushed (Precambrian sandstone)

1.1.2. Granulometric characteristics

The granulometric analysis provides important information for grained materials as the base for material selection in this study. According to the Standard NF EN 933-1 (AFNOR, 1997), the principle of the analysis is passing a representative quantity of sample through a series of sieves with decreasing opening by mechanical shaking. The distribution of grains size is represented by the mass retained between two consecutive sieves. The openings of nominal sieves usually used are: 0.08, 0.16, 0.2, 0.25, 0.315, 0.4, 0.5, 0.63, 0.8, 1, 1.6, 2, 2.5, 4, 5mm.

About 1kg of each sample was dried at 105°C in ventilated oven over night, in order to eliminate the humidity. The dried sample was placed in the first sieve (largest opening) and was sieved through the decreasing opening during 15-20min shaking. The distribution curve

was established by the percentages of the refused mass of each sieve to the total dried sample quantity. This curve provides the access to several characteristics: effective size (D_{10}), average size, uniformity, and fine particles percentage, summarized in Table 2.1.2.

Table 2.1.2: Granulometric parameter definitions

Parameters (unit)	Symbol	Definition	
Effective diameter (mm)	D_{10}	The effective diameter is the opening indicated by the distribution curve at 10% passing.	
Average diameter (mm)	D_m	$D_m = \frac{\sum(m_i D_i)}{\sum m_i}$	With m_i: the refused mass retained on the sieve *i* [M]; and D_i: the average opening of two consecutive sieves [L].
Uniformity coefficient(-)	UC	$UC=D_{60}/D_{10}$	With D_{60} is defined similarly as the opening indicated by the curve of 60% passing sample
Fine particle content (%)	Fine%	The fine particle percentage is the percentage of passed mass though 0.08mm sieve to total sample mass.	

1.1.3. Physical characteristics

Some physical characteristics were determined by laboratory methods, such as volumic mass density, relative density, porosity, and specific area is based on estimated value. These parameters were determined according to Liénard *et al.,* (2001).

❖ *Volumic mass density (real density) (ρ)*

The real density is estimated by placing the sample of known mass slowly into a graduated test tube which has known volume of clear water. This method is rapid and simple however less precise, so the test should be carried out at least 3 times to have an average value. About 300 g of dried material is weighed precisely, and then carefully and slowly poured into the known volume water. The volume moved from V_1 to V_2, and the difference of volume

represents the real volume of dried grains without pores, and the real density ρ (kg/m^3) is calculated by the following equation:

Equation 1: $$\rho = \frac{M_g}{V_2 - V_1}$$

With M_g: mass of dried sample (kg); $V_g=V_2-V_1$ is the volume difference of after and before placing the sand in water, representing the dried grain volume.

❖ *Porosity (φ)*

The porosity can be estimated at the same time with the real density. The total volume (V_t) is measured by graduated test tube for the same dried sample. The void volume is represented the difference between the total dry volume of sample and the volume of the grains:

Equation 2: $$V_v = V_t - V_g$$

Equation 3: $$\varphi = \frac{V_v}{V_t}$$

❖ *Bulk density (apparent density: ρ_{app})*

The bulk density is estimated by the mass and the total volume of sand sample:

Equation 4: $$\rho_{app} = \frac{M_g}{V_t}$$

❖ *Specific surface*

The specific surface is the total surface of grains per volume or mass unity. It depends on the size and the shape of testing material. The specific surface can only be estimated since the measurement of grain shapes is complicated. The specific surface of spherical grains of uniform size is estimated by the average size:

Equation 5: $$A_s = \frac{\pi {d_m}^2}{\frac{\pi}{6}{d_m}^3} = \frac{6}{d_m}$$

For the materials of rounded grains of the various sizes, such as the alluvial sands, the specific surface is estimated by the sum of mass fraction (%) of each size (average opening of two consecutive sieves):

Equation 6: $A_s = 6 \sum \frac{X_i}{D_i}$

The above equation can be only served to estimate the specific surface of rounded grains. However, even the river sands sometime can hardly considered being rounded, and for crushed sands, the shape is much more irregular. Lakel (1995) has proposed a modification by introducing a shape factor φ into the equation:

Equation 7: $A_s = \frac{6}{\varphi} \sum \frac{X_i}{D_i}$

With φ=1 for sphere and φ=0.7-0.95 for sands. In this study, the factor is 0.95 for river sands and 0.7 for crushed aggregates, and the specific surface is expressed on m^2/kg, by dividing real density: $A_s\ (m^2/kg) = A_s\ (m^{-2})/\rho\ (kg/m^3)$.

❖ *Grains shapes observation and image analysis*

Attempt has been made to access the grain shapes of two different types of sands in this study. Observations by camera and by microscope showed a poor representation of the entire sand sample. The images of the bulk sample (dried, fine particle eliminated) were taken by camera, and the some grains of certain sizes (0.25-0.4mm; 0.6-0.8mm) have been sampled after the sieving tests. The microscopic approach is based on the studies (Cho *et al.*, 2006). Pictures of aggregates were processed by ImagJ software. After an 8 bit and a binary conversion the picture scale was set and the analysis of particles were run with a specific plug in. Filter were used with a limitation of size (0.1 to 4 mm) and circularity (>0.75) for a good isolation of particles. The isolation process is presented in Figure 2.1:

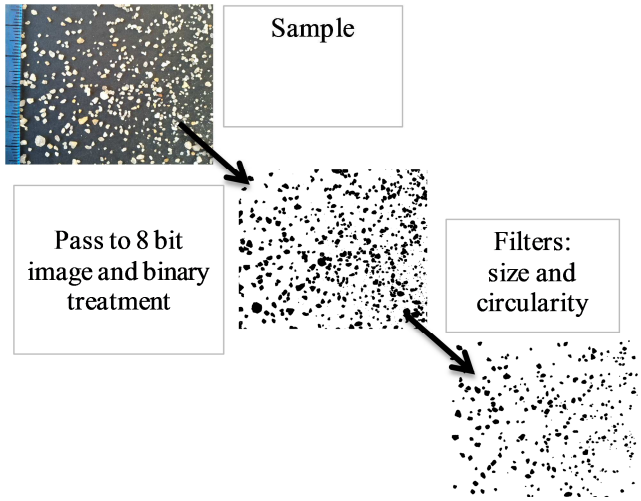


Figure 2.1: Image processing of form parameters analysis for filter materials

1.1.4. Chemical characteristics

Sands and crushed aggregates are considered as chemical inert and stable materials. However, the surface can contain exchangeable cations such as: K^+, Na^+, Ca^{2+}, Mg^{2+}, and Al^{3+}that may alter the ionic strength. The mineralogical analysis was effectuated by local laboratory IDAC (Inovalys, Nantes). The nominal method of released elements does not exist. In this study, a procedure is proposed in order to study the contents of certain released elements, and four elements have been analyzed by Atomic Emission Spectroscopy.

❖ *Released cations*

The materials are dried and sieved through 0.08mm. Three different masses were sampled, placed into 50mL plastic tubes, and washed with 20mL ultrapure water for 3 times. The experimental conditions are summarized in Table 2.1.3:

Table 2.1.3: Experimental conditions for released cations of selected sands

Sample treatments	Dried sieved aggregates (>0.2mm)
Temperature	Ambient, 25°C
Solid/liquid	5g/20mL; 10g/20mL; 15g/20mL
Mixing condition	Mixing table, 200tr/min, overnight
Liquid phase sampling	Settled for 2 hours, 15mL filtrate of 0.45µm
Detected cations by ICP-ES (Shimadzu)	K^+, Na^+, Mg^{2+}, and Ca^{2+}

❖ *Mineralogical analysis*

The mineralogical analysis was conducted by local laboratory IDAC (Inovalys, Nantes). The materials were attacked by nitric acid under high heat or microwave and dissolved into the mix solution of $LiBO_2$ and $Li_2B_4O_7$. The major elements were quantified by Atomic Absorption Spectroscopy.

❖ *Calcareous (Limestone) contents*

The calcareous (limestone) content measurements were also carried out by IDAC (Inovalys, Nantes).

1.2. Process design: filtration reactors

The process of filtration reactors is composed of three parts: mixing and feeding, main pilot and evacuation. An overall view of the pilot is simplified in Figure 2.2:

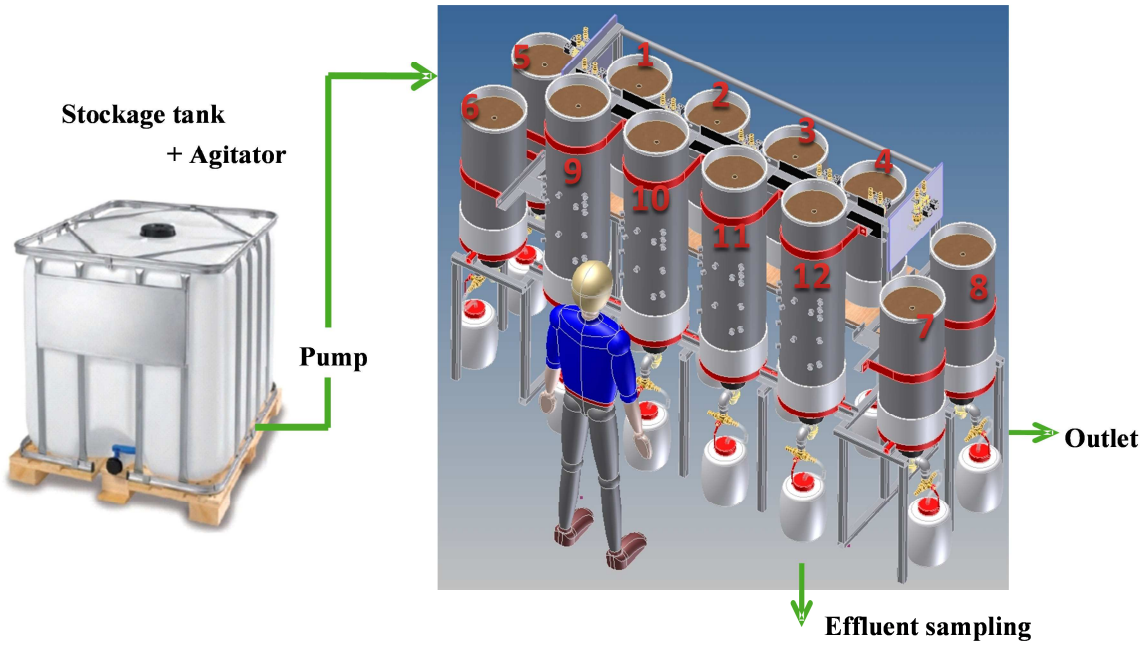


Figure 2.2: Simplified overall view of the experimental pilot

❖ *Feeding tank*

Due to the distance of septic effluent sampling site, the feeding tank was also served as a mixed storage tank. This tank had a capacity of $1m^3$ which insures one week of feeding. The septic effluent was renewed once a week and the tank was continuously mixed. The feeding water was pumped by a peristaltic pump.

❖ *Filtration pilot and cylinder reactors*

Four selected packing materials, including two types of river sands (RS1 and RS2), and two types of crushed aggregates (CA1 and CA2) have been tested, and their position arrangements in the reactors are presented in Table 13. The pilot is composed by 12 cylinders of three different heights: 15cm (Reactors 1-4), 30cm (Reactors 5-8) and 70cm (Reactors 9-12). The diameter for all the columns was identical: 30cm. For the reactors of 70cm (9-12), sampling ports were installed at 5, 10, 15, 30, 45cm. The inclined ports with half tube were supposed to serve effluent receptions which were not achieved during the operation, so these ports were also served for sand sampling. The Figure 2.3 shows the positions of the sampling ports:

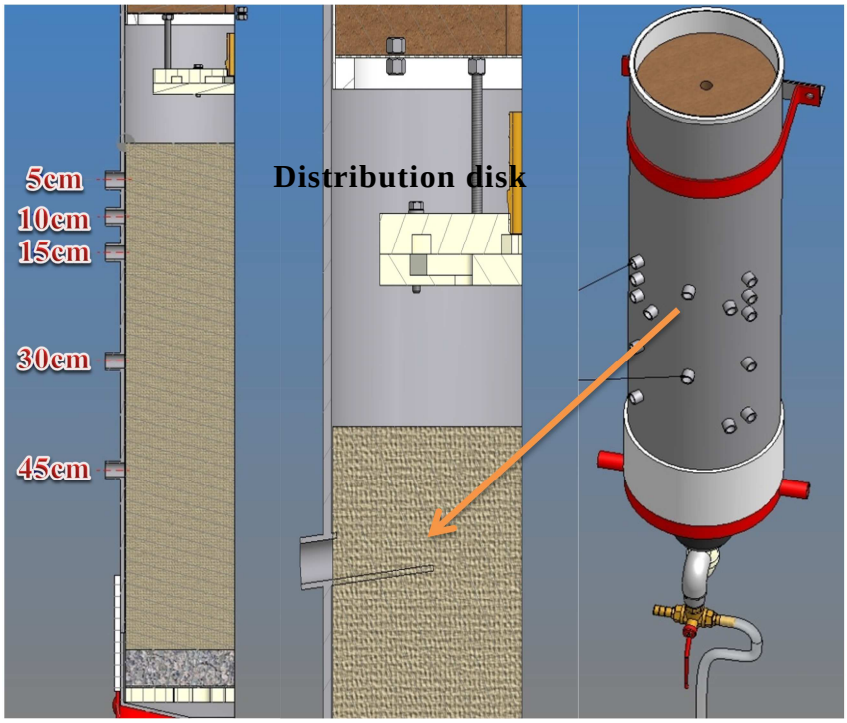


Figure 2.3 : Sampling ports positions of 70cm columns

1.3. Experimental procedure of filtration reactors characterization

1.3.1. Hydraulic characteristics

It is important to know hydraulic characteristics of filter materials for on-site wastewater treatment use. The estimations of hydraulic conductivity and water retention capacity are carried out in this study. Two experimental devices were installed for hydraulic conductivity and water retention characterization.

1.3.1.1. Estimation of saturate hydraulic conductivity at laboratory scale

The method for saturated hydraulic conductivity determination is normalized for on-site soil tests (NF X30-441, AFNOR, 2008). A simplified method for rapid determination at laboratory scale is proposed by Grant (Liénard *et al.*, 2001). This method allows the determination of saturated hydraulic conductivity (k_s, *m/s*) through the measurements of the infiltration time (Grant time: t_g). The calculation is based on the Darcy's law:

Equation 8:
$$k_s = \frac{0.0553}{t_g} = \frac{H_{s.exp}\ln\left(\frac{4V_{exp}}{\pi D_{exp}^2 H_{s.exp}}+1\right)}{t_{exp}}$$

With $H_{s.exp}$is the material layer height in experimental condition (m); $V_{s.exp}$is the water volume poured in the sand (m^3);$D_{exp}$is the diameter of sand layer surface (m); and t_{exp} is the passing time of this volume of water (s).

The experimental device proposed in this study is according to Liénard *et al.*, (2001). The sand was fed with clear water several times until meet saturated condition (thin constant film of water on the surface). Then 500mL clear water was poured slowly on the surface and the passing time of this 500mL water was noted. This measurement should be at least repeated for 5 times to obtain an average value. According to the authors, Grant infiltration time should be fitted the threshold between 50s and 150s, corresponding to k_s between 3.7×10^{-4} and 1.1×10^{-3}. The Figure 2.4 shows the experimental conditions of this test:

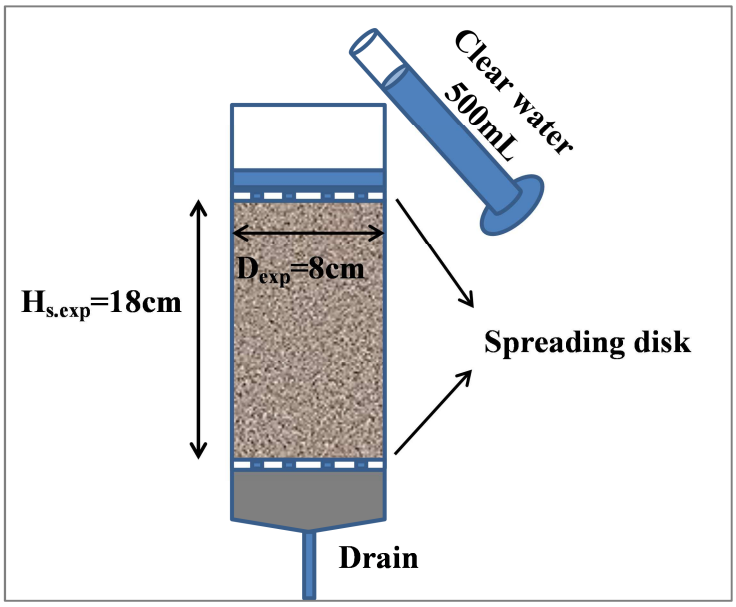


Figure 2.4: Experimental device for hydraulic conductivity determination by Grant method

1.3.1.2. Water retention capacity

Beside the capacity of conducting liquid, grained materials also have the capacity of retaining certain portion of liquid under the effect of capillary pressure after the drainage. This capacity is characterized by two methods proposed by Mohammedi (1998): method of permanent flow rate, and drainage method. The two methods provide respectively the access to the total volume retained in porous media and the volume retained after the drainage (stagnant water).

❖ *Method of permanent flow rate*

Several columns of 70cm height (material layer height), 8cm diameter are used in this study. The column is packed with dried material sample, and the column is fed with a hydraulic load of 2cm/min which corresponding to a flow rate of 10mL/min. The flow rate at the outlet of the column is noted as function of the time. When the flow rate is stable and constant, the feeding is stopped.

❖ *Drainage method*

The flow rate decreases after stopping the feeding. The recording of flow rate is continued until no water comes out from the column. The Figure 2.5 shows the principle of the two methods:

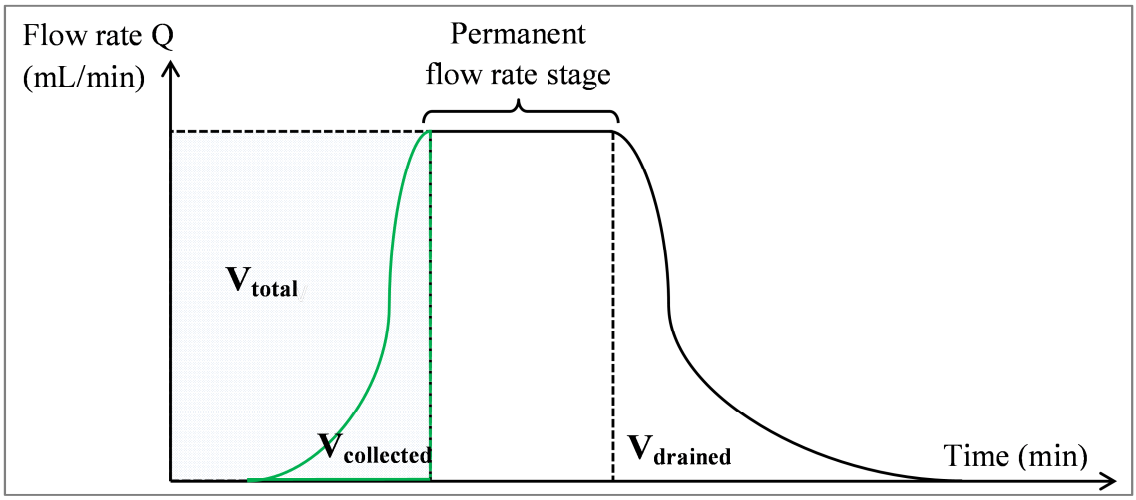


Figure 2.5: Schematic figure of water retention test

The total water volume is represented by the difference between the total fed water volume (flow rate × time obtained constant flow rate, area of rectangular) and the collected volume (area included in the green line in the figure). The capacity is expressed either by the ratio between the V_{total} and the volume of material (C_v%) or between the M_{total} and the mass of material (C_m %). The massive capacity is characterized by the measurements of humidity (mass loss at 105°C). The total water involved in the sand column includes the drainable water and retained water. The volume of drained water is represented by the area under the curve after stopping the feeding ($V_{drained}$).

1.3.2. Reactor characterization

In an unsaturated filtration rector, except the solid phase provided by the filter media, characterized by the packing materials, the liquid phase and the air phase also present and both involve in the treatment process. In this study, the hydrodynamic characteristics and oxygen gas contents in reactors (or in certain reactors) have been examined.

1.3.2.1. Hydrodynamic characteristic

The flow characteristics are often represented by the hydrodynamic behavior. The hydrodynamic behavior impacts the functioning of filtration reactor. The initial hydrodynamics depends on the intrinsic characteristics of packing materials, operating conditions and also the reactor itself. This parameter is often characterized by the distribution of residence time which gives access to the average hydraulic residence time (HRT)of filtration reactors.

- *Tracer choice*

The choice of the tracer should respond to the following requirements: 1) detectable of small quantities; 2) neutrality by bioreactors; 3) no nuance for the biomass; 4) no retention in the biomass.In this study, lithium chloride (LiCl) was used as the tracer.

- *Injection*

The quantity of tracer injected into the systems varies from case to case. For packing material systems, the tracer quantity injected is supposed to be 0.5-1g of Li^+ per m^3 material which his suggested by Maillard (1998). In this study, 1g Li/L solution was prepared. 20mL and 30mL solution were injected respectively for 12cm/day and 20cm/day loading rate. The reactors were dosed by 10 batches per day for both loading rates. The tracer was injected with certain batch.

- *Sampling*

The effluents were sampled right after the injection. Before the peak detected, more samplings were effectuated. After the passing of the peak, the sampling frequency was decreased.

- *Detection*

The detection of Li concentration in effluents was realized by atomic absorption spectroscopy (SpectrAA 220, VARIAN). The detection limit was between 0.5 and 8mg Li/L, so the dilutions were necessary for certain samples.

❖ *Establishment of residence time distribution (RTD) curves and average residence*

The establishment of RTD curves is based on the calculation of $E(t) = C/C_0$ as function of time *t*. The calculation of the function C/C_0 is shown in the following equation:

Equation 9: $$E(t) = \frac{C_i}{\sum C_i \Delta t_i}$$

With C_i(mg/L) is the tracer concentration of sampling moment t_i; and Δt_i is the delay between two consecutive samplings.

The average hydraulic residence time (HRT)$t_s$is calculated as:

Equation 10: $$t_s = \frac{\sum C_i t_i \Delta t_i}{\sum C_i \Delta t_i}$$

1.3.2.2. Estimation of oxygen gas variation

During each batch of feeding water pumping, the oxygen gas level fluctuates with the passage of the liquid flow. The estimation of oxygen gas variations was effectuated with 70cm reactors at 10cm layers by optical oxygen sensor (OXROB10, Pyroscience sensor technology). The excitation signal was sent at the wavelength of 620nm and the emission signal was 760nm processed at by optical oxygen meter (Fire Sting O2, Pyroscience sensor technology). The data was recorded every 10 seconds during 24h. The detection limit of oxygen sensor is 0.02% O_2.

1.4. Results and discussions

1.4.1. Material characteristics

In this section, the main characteristics of selected filter materials are discussed, including granulometric, physic and chemical characteristics. These characteristics are intrinsic properties of materials, invariable during the operating.

1.4.1.1. Granulometric characteristics

The size distribution curves of selected materials are presented in Figure 2.6 (river sands) and Figure 2.7 (crushed aggregates). These curves are compared to the minima and maxima of French standard DTU 64.1.

The selected materials including two river sands and two crushed aggregates are all in the proposed range of filtration use according to DTU.64.1. From the distribution curves, it appears that RS1 is relatively fine sand and RS2 is very coarse. On contrary, the crushed aggregates are well graded and heterogeneous in size, presenting both fine and coarse particles. With the help of size distribution curves, several parameters can be defined for each material. The detailed information of these parameters is provided in Table 2.1.4:

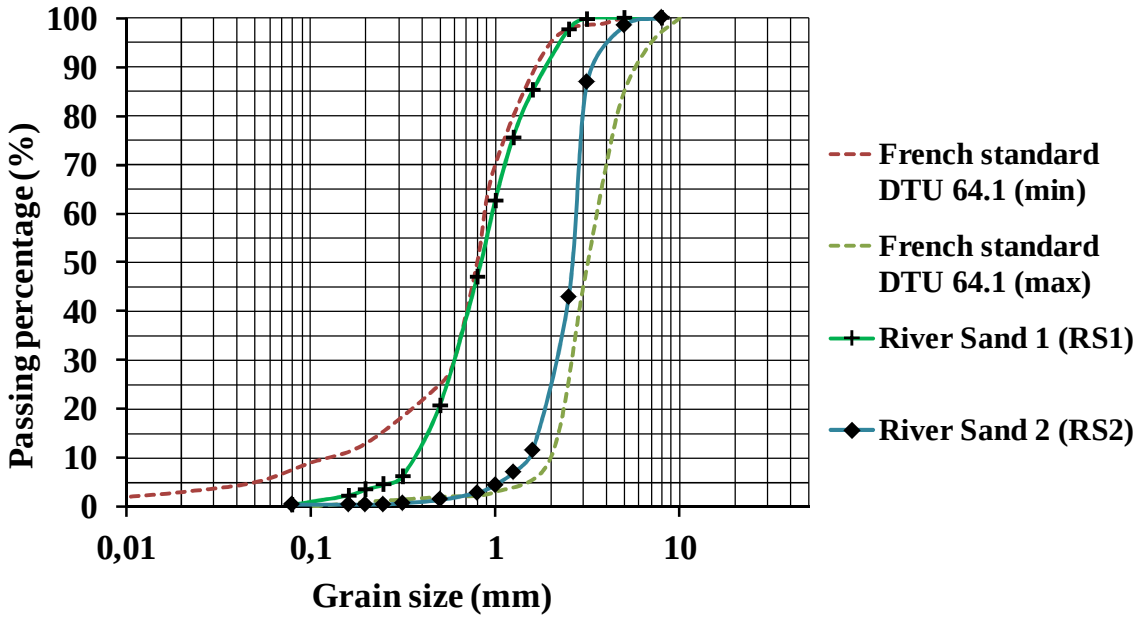


Figure 2.6: Size distribution curves of river sands (RS1 and RS2)

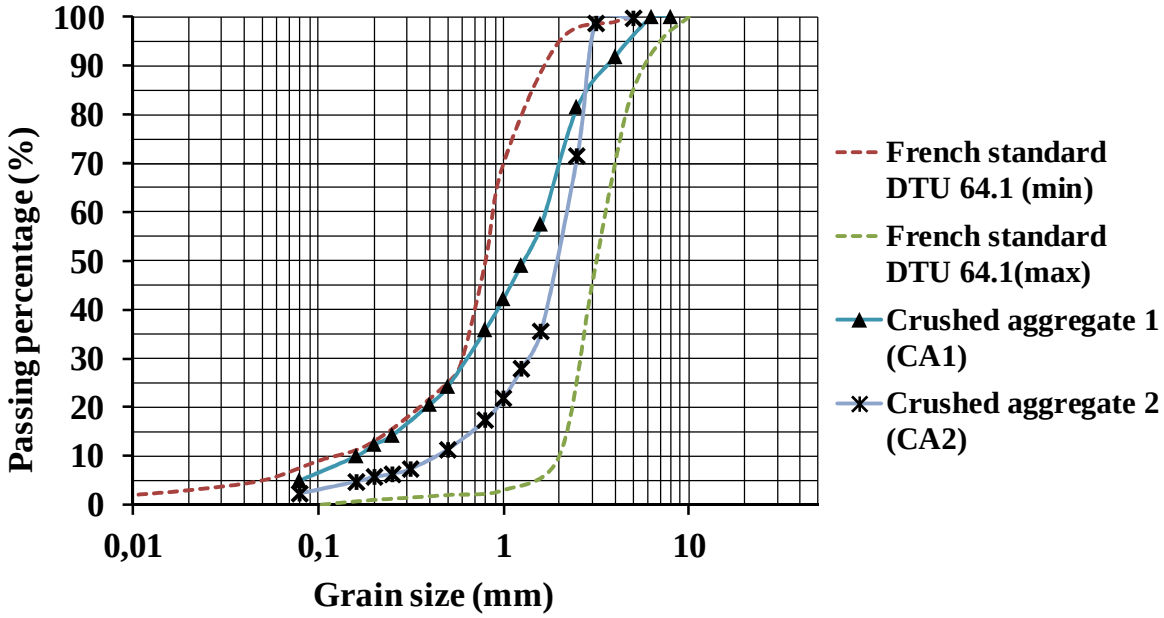


Figure 2.7: Size distribution curves of crushed aggregates (CA1 and CA2)

Table 2.1.4: Granulometric characteristics of filter materials

	RS1	RS2	CA1	CA2
Material nature	Loire river	Loire river	Crushed	Crushed
Effective size D_{10} (mm)	0.38	1.60	0.17	0.44
Average diameter D_m (mm)	0.82	2.26	1.36	1.6
Uniformity coefficient (D_{60}/D_{10})	2.78	1.75	10	5
Fine particles (<0.08mm)%	0.4%	0.5%	5%	2.4%

❖ *Diameter and uniformity:*

The effective diameter of each sample was determined from the granulometric curves (Figure 2.1; 2.2). The results showed that the river sand: RS2 were coarse sand. Even though the D_{10} of RS2 was in accordance with DTU 64.1, it exceeded the limits of filtration sand choice recommended by Liénard *et al.,* (2001) who indicated that the sand of effective diameter between 0.2 and 0.4mm conducted practically better biological treatments. In this case, RS1, CA1 and CA2 are in accordance of this requirement. The CA1 presents fine effective diameter (D_{10}=0.17mm) but relatively large average diameter (D_m=1.36).

The uniformity coefficients show that the grain size distributions of crushed aggregates (CA1 and CA2) are more heterogeneous than river sands, especially CA1. Liénard *et al.,* (2001) suggested in their study that an UC inferior of 4 would suit better for filtration treatments and also indicated that crushed aggregates tend to be well graded in size (9~27). The river sands in this study show accordingly satisfied uniformity. Even though RS2 has a better uniformity, the grains have relatively larger sizes.

❖ *Fine particles content:*

Crushed aggregates show higher content of fine particles (size inferior of 0.08mm), especially CA1. Liénard *et al.,* (2001) indicated that the permeability decreased with the increase of fine

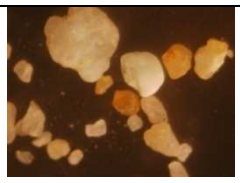
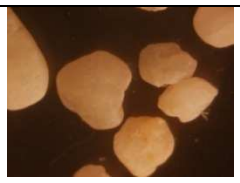
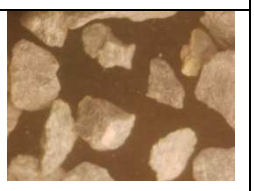
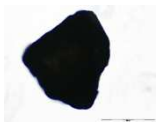
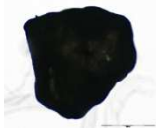
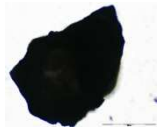
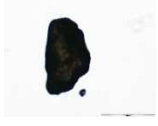
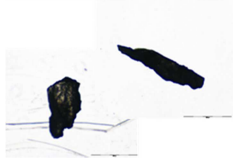
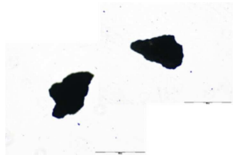
particle content and the percentage should not be superior of 2.5% for filtration process. During the packing, materials are washed and compacted by water in order to eliminate the dust or very fine particles, even though they are not totally removed.

From the effective diameters and uniformity coefficients, the RS1 and CA2 are more in accordance to the material recommendations for filtration bed packing and CA1 can be considered as the extreme situation of granulate materials that their consequences in filtration process should be fully examined. The results show that the RS1 is more appropriate for filtration use. In this study, this material is considered as the reference material. The crushed aggregates show great differences in the size distribution, and their consequences of applying these materials may lead to various functioning.

1.4.1.2. Morphologic characteristics of materials

Several physical parameters were estimated with laboratory methods and several images of material grains of various sizes are taken by binocular or microscopic capture. The forms of material grains (circularity and roundness) were studied by the image analysis through Software ImagJ. The results are summarized in Table 2.1.5:

Table 2.1.5: Photos of material grains of different size taken by binocular or microscope

Materials	RS1	RS2	CA1	CA2
Sample photo by binocular				
0.8-1mm				
0.25-0.4mm				

The images give direct descriptions of material particles. Even though the analysis on form parameters (roundness and circularity) does not show significant differences, the sample

photos indicated that the river sands (RS1 and RS2) are generally round, regular particles and have smooth edges, even for the smaller particles. On the contrary, the crushed aggregates present very irregular, oval or long forms and have angular and rough edges. The smaller particles of crushed aggregates present more imperfections. The differences on particle forms between two kinds of materials presumably lead to different arrangements among the grains, thus may impact the filter functioning or biomass development onto these materials. The results of physical and morphological parameters are summarized in Table 2.1.6:

Table 2.1.6: Physical and form characteristics of filter material (Average value of 5 tests for physical characterization)

	RS1	RS2	CA1	CA2
Real density (kg/m^3)	2525 (±42)	2564 (±164)	2438 (±111)	2646 (±51)
Bulk density (kg/m^3)	1738 (±9)	1625 (±108)	1576 (±39)	1552 (±61)
Porosity [min, max] (%)	[30%; 33%]	[37%; 41%]	[38%; 41%]	[38%; 44%]
Specific surface (m^2/kg)	4.04	1.18	2.78	2.88
Circularity (-)	0.849 (±0.060)	0.819 (±0.037)	0.824 (±0.088)	0.800 (±0.109)
Roundness (-)	0.74 (±0.11)	0.76 (±0.10)	0.73 (±0.15)	0.67 (±0.15)

The materials have similar physical properties, and the real density and bulk density are in the range of sandy soil: 2600 and 1600kg/m^3 (Hillel, 1988). The porosity is an indicator of relative volume of pore space in the minerals. Except for the fine river sand (RS1), the coarse sand (RS2) and crushed aggregates (CA1 and CA2) have slightly higher porosity, but the porosities of four materials are all in the range of sandy soil: 30-60% (Hillel, 1988). The physical characterization can only provide basic and loose information that indicate that river sands and crushed aggregates physically showed no significant differences except the roundness. The image analysis shows the crushed aggregates are more angular, less spherical (less roundness and circularity) and is in agreement with previous studies (Wanko *et al.*, 2005; Cho *et al*, 2009).

1.4.1.3. Chemical and mineralogical characteristics

The river sands are considered as inert materials and their chemical characteristics do not impact the filter functioning. The crushed aggregates, as exploited from rocks are also chemically stable. Authors have indicated that crushed aggregates show slightly difference in mineral composition (Wanko *et al.,* 2005).

The mineral compositions can affect the surface properties of the materials, such as the cations associated onto the grain surface. The chemical properties are particularly concerned in the study of the materials for wastewater treatments because the interactions taken place at liquid/solid interfaces may influence the adhesion of bacteria and the abatements of certain pollutants. The mineral compositions of filter materials are presented in Table 2.1.7; four cationic elements released by contacting with ultrapure water at pH=7 are summarized in Table 2.1.8.

Table 2.1.7: Mineral compositions and calcareous (limestone: $CaCO_3$) contents of filter materials

mg/kg	RS1	RS2	CA1	CA2
Ca	<5	<5	795	7002
Mg	74	17	1535	11022
Na	<5	<5	295	537
K	1370	139	2356	4323
Fe	<1000	<1000	5576	32978
Al	17075	1507	38800	59383
Si	431166	460834	391779	256906
	RS1	RS2	CA1	CA2
$CaCO_3$ (%)	<0.5	<0.5	1.3	1.7

Table 2.1.8: Released cations in water from the filter materials

	RS1	RS2	CA1	CA2
Ca (mg/kg)	4.30	2.28	10.43	28.72
Mg (mg/kg)	0.52	0.33	1.37	3.64
Na (mg/kg)	0.98	0.97	6.91	4.61
K (mg/kg)	1.47	0.78	7.53	4.63

The results show that both river sands and crushed aggregates are silicon based materials but crushed aggregates are more heterogeneous in mineral compositions due to the origins of materials. The two river sands present similar compositions since both materials are exploited from Loire River. The crushed aggregates are sandstone origins (CA1: Feldspathic sandstone and CA2: Precambrian sandstone). According to Mcbride (1963) the feldspars are often pink to red in color, calcite or quartz based and angular in forms and tend to weather to clay minerals under humid conditions but depend on the depositional environment; Pettijohn (1987) has also pointed out that the feldspars are more alkaline and present higher contents of Na, K and Ca. The material CA1 in this study can be recognized in several characteristics of feldspars. The release cations which were in accordance with grain compositions showed higher released Ca and Na with crushed aggregates. These elements are susceptible contributing some anions precipitation (ex: phosphate). The presence of metal elements at the surface of feldspars is also associated with their oxides (-O) or hydroxides (-OH) which contributes as well as the precipitation of some anions.

1.4.2. Reactors characteristics

1.4.2.1. Hydraulic characteristics

The water movement characteristics in a filter medium are one of the important properties in filtration beds, which basically depend on the filter materials. The filter material has the capacity of conducting the liquid phase flowing through the media known as the hydraulic conductivity, which can be estimated trough the infiltration test by measuring the time of infiltration. The filter material also has the capacity of retaining the liquid in the media and the liquid phase attached to the grain surface loosely or firmly bounded. This capacity is estimated by the water retention test. The results of and infiltration time (t_s) and estimated saturated hydraulic conductivity (K_s) are presented in Table 3.6. The results of capacity of water retention: volumic capacity (C_v) and massive capacity (C_m) are summarized in Table 2.1.9.

Table 2.1.9: Infiltration time and estimated hydraulic conductivity at saturation and water retention capacities of filter materials

Hydraulic characteristics	RS1	RS2	CA1	CA2
t_s (s)	62	20	170	67
K_s(m/s)	8.95×10^{-4}	2.77×10^{-3}	3.25×10^{-4}	8.25×10^{-4}
Water retention capacity	RS1	RS2	CA1	CA2
Cv (%)	14.6%	2.1%	20.0%	15.5%
Cm (%)	8.4%	1.3%	12.7%	10.0%

The coarse river sand (RS2) shows the highest hydraulic conductivity and also the lowest water retention capacity. The crushed aggregate especially CA1 shows relatively low hydraulic conductivity which suggests that the flow is relayed in this material due to the differences in size and shape of particles. Authors have indicated that the estimated hydraulic conductivity should be ranged between 1.1×10^{-3} and 3.7×10^{-4} for filtration use. The water retention capacity also shows that CA1 holds more water and retains within the medium.

From the above, the differences in materials intrinsic characteristics are not only observed in size distributions, but also in forms and mineral compositions which indicate the problematic of altering filter materials from sands to crushed aggregates. The consequences of applying filtration reactors based on these materials can be dramatic.

1.4.2.2. Hydrodynamic behaviors

The hydrodynamic behaviors in filtration reactors are one of the most important parameters that govern the purification process the development of biomass. The reactors of 70cm and 30cm are the main reactors in this study and the flow characteristics are studied as hydrodynamic behavior by the distribution of hydraulic residence time. The average HRT of each reactor are summarized and the oxygen gas variation in Table 2.1.10.

Table 2.1.10: Average HRT in 30cm and 70cm reactors

Average HRT (hour)	RS1	RS2	CA1	CA2
12cm/day, 30cm	23	8	32	20
12cm/day, 70cm	35	-	93	-
20cm/day, 70cm	12	-	48	-
Oxygen gas variation (oxy% in air)	[11; 20]%	-	[19.2; 19.8]%	-

The hydrodynamic behavior of a filtration reactor is not only affected by the packing materials, but also the operational parameters and the reactor size. The results of 30cm reactors at 12cm/day show in the accordance with the hydraulic conductivities and water retention capacities of the filter materials. The coarse river sand (RS2) which is very permeable material shows low water residence time, poor water retention capacity and suggests that the solutes stay relatively shorter time in this material. As the contrary, the CA1 presents higher average HRT value, which suggests the existence of the solute exchange or interaction between the liquid phase and the material. As observed with granulometric analysis, this material contains more fine particles, even though the washing took place during the packing, some smaller or fine particles still stay in the system. Authors also suggested that the crushed aggregates with very angular, irregular particle shapes tend to form more tortuous flow paths and make the paths longer than in round, smooth materials (Wanko *et al.*, 2005). The 70cm reactors show much higher HRT volume in CA1. The HRT values decrease in 70cm reactors with higher hydraulic loading (20cm/day) for both RS1 and CA1. The oxygen gas sensor were applied one time for the two materials and the oxygen gas variations showed that in RS1, there was saturated/unsaturated phases during the batch inlet, but in CA1, no alternations of phase have been found and always in unsaturated conditions.

Conclusion:

A partir de cette étude comparative des caractéristiques propres des matériaux et des réacteurs, les agrégats concassés se différencient surtout des sables roulés par leur distribution de tailles de grains. D'autres paramètres peuvent éventuellement influencer les propriétés hydrauliques et hydrodynamiques des réacteurs.

Les différences en répartition des tailles entre les deux types des matériaux sont provoquées par la nature de la source du matériel et la procédure de l'exploitation et de la production en carrières. Les agrégats issus du concassage de la roche massive montrent une distribution des tailles hétérogènes (Coefficient d'uniformité ≥ 5, $CU<3$ pour les sables roulés). Ils possèdent également des particules fines du diamètre <0,08mm qui sont induites par des broyages entre des grains pendant le concassage et le transport (Fines%>2%). Par contre, les sables issus de l'érosion en rivières, montrent une stabilité structurelle (Fines%<0,5%). Des différences sont également observées sur la forme des grains. Les agrégats concassés présentent des formes anguleuses, allongées et irrégulières. L'hétérogénéité des grains en taille et en forme est donc liée à la source des matériaux. Les sables roulés ont la même origine (La Loire) et à base de silice. Les agrégats de roche présentent différentes compositions, ils sont notamment plus riche en Ca, Fe, Al…

Par conséquence, l'hydraulique du milieu se différentie pour les types des matériaux. La présence de particules fines, surtout pour CA1, induit une conductivité hydraulique plus faible que d'autres matériaux, et en même temps, une rétention en eau plus importante : le profil de la répartition de l'eau sur la longueur du lit montre des différences de répartition de la phase liquide :

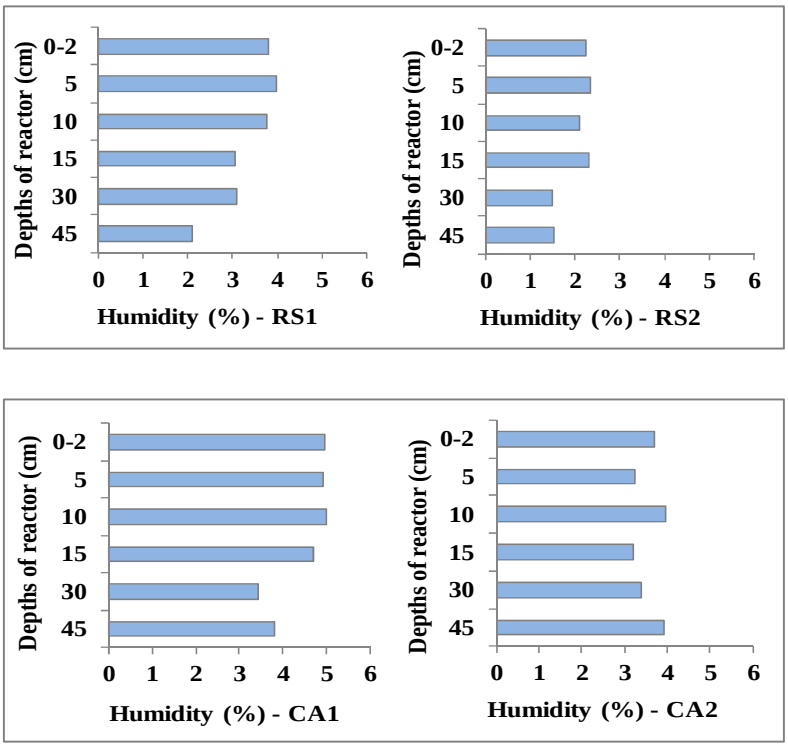


Figure 2.8: profil de répartition de l'eau retenue

L'eau retenue par le filtre représente de l'eau capillaire et pour une faible proportion de l'eau de structure. Les agrégats de roche peuvent avoir des porosités internes différentes de celles des sables de rivières. Les formes anguleuses peuvent également provoquer un arrangement différent que les sables qui possèdent des formes plus régulières et donc créer des empilements moins régulier et une tortuosité de lit supérieure. Ces différences entrainent une hydrodynamique différente dans les réacteurs et en particulier des écarts dans les temps de séjour. L'hydrodynamique va influencer le renouvellement et la répartition du substrat, qui finalement impactera l'implantation et l'évolution de la biomasse.

Pour mettre en évidence d'éventuels impacts de la nature des matériaux de garnissage, un suivi des efficacités épuratoires vis-à-vis du carbone de l'azote et du phosphore est ensuite proposé.

Chapter 2: Study of purification efficiencies with different filter materials

Introduction:

Le chapitre précédent a permis de mettre en évidence des comportements hydrodynamiques différents en fonction du type de matériaux (deux sables de rivière et deux agrégats concassés, nous avions choisi d'observer l'impact des matériaux sur le fonctionnement du procédé sur une durée suffisamment longue pour être dans un état stationnaire apparent (360 jours). Les paramètres mesurés seront classiquement les MES, C, N et P.

L'efficacité épuratoire donnera une vision globale de l'impact des matériaux puisque plusieurs acteurs interviennent dans la dépollution : le matériau et la biomasse.

- Les caractéristiques morphologiques des matériaux sont au moment de la mise en place du procédé :
    i. *via leur arrangement dans le réacteur, directement responsables de la rétention des particules solides de l'effluent*
    ii. *via leur surface spécifique, susceptibles de permettre une colonisation différente du réacteur*
- L'hydrodynamique influencera en particulier la mise en place du biofilm par rapport à son accès au substrat alors que la nature chimique du matériau est susceptible d'influencer l'adhésion et le développement du biofilm, mais aussi une précipitation éventuelle des phosphates (Zanini *et al.*, 1998; Robertson, 2003).

Par ailleurs, l'impact sur la biomasse sera abordé par une estimation de la flore autotrophe nitrifiante et hétérotrophe à différentes hauteurs du réacteur.

Afin d'approfondir les comparaisons d'efficacité d'abattement, différentes conditions opératoire sont mises en place :

- Différentes hauteur de réacteur : 15, 30 et 70 cm (30 étant la hauteur relative à l'étude du suivi des abattements sur l'ensemble de la période des 360 jours)
- Différentes charges hydraulique pour le réacteur de 70 cm de hauteur : 12 et 20 cm/jour.

2.1 Experimental procedures of purification efficiencies analysis

The feeding water and treated effluent characterization was carried out every 2 weeks in order to follow the filters purification performance. Both feeding water and treated effluents were sampled and stored at 4°C.

2.1.1. Feeding water distribution, treated effluent sampling and dosing schedule

❖ *Feeding water distribution and treated effluent evacuation*

The feeding water was spread by the distribution disk, which was punched of 15 holes. The homogeneous distribution is difficult to achieve. The evacuation of treated effluents was achieved by the 3 way valves which provided the access to the effluent sampling tanks and to the drainage.

❖ *Dosing planning*

The program of pilot feeding: openness and close of peristaltic pump and electric valves were controlled by computer, so these parameters could be altered or deposited. Filtration reactors were supplied discontinuously by batches with septic effluent in order to keep the unsaturated conditions. The hydraulic charge described by the thickness of effluent above material layer per day (charge: 12cm/day or 20cm/day corresponding to 8.5L/day or 14L/day) were fractionated by 10 batches at each column during 24 hours. The Table 2.2.1 summarizes the dosing planning throughout the operation stage:

Table 2.2.1: Hydraulic loading, packing materials and feed-rest frequencies

Reactor Number in Figure 2.2	Hydraulic loading (cm/day)	Packing materials	Dosing frequency (batches/day)
1	12	RS1	10
2	12	RS2	10
3	12	CA1	10
4	12	CA2	10
5	12	RS1	10
6	12	RS2	10
7	12	CA1	10
8	12	CA2	10
9	12	RS1	10
10	20	RS1	10
11	12	CA1	10
12	20	CA1	10

2.1.2. Physico-chemical parameters analysis

The purification by sand filtration mainly concerns the treatments of particulate pollutants, organic carbon pollutants, nitrogen pollutants and phosphorous pollutants. The treatment efficiency is calculated as:

$$\Delta\% = \frac{C_0 - C_i}{C_0} \times 100\%$$

The tested parameters and their methods are presented in Table 2.2.2:

Table 2.2.2: Analytic methods of physico-chemical parameters of feeding water and treated effluents

Physico-chemical parameters	Method	Testing range
pH	Multimeter	1-14
Conductivity (µS/cm)	Multimeter	
COD (mgO/L)	Merck COD Vials Kit	150-1500mgO/L; 10-150mgO/L
N_{total} (mgN/L)	Merck Total N Vials Kit	10-150mgN/L
NH_4^+ (mgN/L)	Merck Ammonium 100 Tests	2.6-19.3 mgNH_4/L
NO_3^- (mgN/L)	Merck Nitrate 200 Tests	0.4-110.7mg NO_3/L
PO_4^{3-} (mgP/L)	Merck Phosphate 100 Tests	1-100mgP/L

Table 2.2.3: Septic effluent characteristics during long-term monitoring

Reactor height	Materials			
	River sands		Crushed aggregates	
15cm	RS1		CA1	
30cm	RS1	RS2	CA1	CA2
70cm	RS1	RS1	CA1	CA1
Dosing conditions	12cm/day or 20cm/.day fractionated by 10 batches/day			
	Septic effluent characteristics (Average value and [min, max])			
Parameters	**Average value**		**[min; max]**	
pH (5 tests)	7.1		[6.6; 7.5]	
TSS (mg/L, 18 tests)	39 (±11)		[20; 66]	
VSS (mg/L, 1 test)	23		-	
COD (mgO/L, 18 tests)	372 (±100)		[231; 572]	
Tot-N (mgN/L)	81		[58; 95]	
NH_4^+ (mgN/L, 22 tests)	46 (±21)		[20;77]	
NO_3^- (mgN/L, 22 tests)	<2.3		[0; 2.3]	
PO_4^{3-} (mgP/L, 14 tests)	9		[7.2; 12.7]	
Revivifiable aerobic flora* (37°C) (CFU*/100mL)	5.1×10^5		[9.5×10^4; 1.1×10^6]	

*Revivifiable aerobic flora (NF EN ISO 6222); *CFU=Colony Forming Unit

2.1.3. Feeding water origin and characteristics

The packed reactors were fed with septic effluent during the long-term monitoring. The wastewater collected from La Chapelle sur Erdre (3km from Nantes, 33.42km^2, 17 709 habitants) was pretreated in three septic tanks for 5 days in order to separate the liquid/solid phase. The settled sludge is rested over 2 years. The pretreated effluent from 3 septic tanks is called septic effluent and collected from a decanter before pumped to the reactors. The main characteristics of septic effluent are presented in Table 2.2.3.

2.1.4. Cells extraction and assessment

❖ *Sampling*

The cells have been extracted from material samples and revivifiable aerobic heterotrophic flora and autotrophic flora (nitrifying bacteria) counts were carried out based on the culture of harvested cells. Due to the coarseness of river sand 2 (RS2), this test was only carried out with three other materials from 3 layers of reactors: 0~5cm, 10~15cm and 30cm. The fresh materials were sampled and processed as soon as possible.

❖ *Cell extraction*

10g of sample was washed with 100mL of 9% NaCl solution and the washed and the cells were extracted by sonication (sonication bath, 60Watts, 15 min at 4°C). The samples received same ultrasound energy by putting in concentric circle with same separated space from the bath wall (Bigois, 1985; Lakel, 1998).

❖ *Assessment*

The extracted cell suspend was diluted with 9% NaCl solution and inoculated to corresponding culture mediums.

- The revivifiable aerobic flora (heterotrophic) was cultured on solid medium of plate count agar (PCA). 1mL of cell suspension was seeded. The incubation was effectuated at 30°C during 3 days.
- The procedure of nitrifying autotrophic flora assessment was adapted from the work of Aragno (1974). The liquid mineral medium enriched with ammonium ions was used and the composition is summarized in Table 2.2.4. 0.2mL of cell suspension was seeded in 1.8mL microplate. After 3 days of incubation at 30°C, the presence of nitrites was recovered by adding sulfuric acid and alpha-naphtylamine (reagent Nit 1 and Nit 2 of analytical profile index: API20E) in each cupule. In case of the absence of nitrites, zinc powder was added in order to recover the nitrates. Five copies were effectuated for each dilution.

Table 2.2.4: Liquid medium additives for aerobic autotrophic flora count (MPN*)

Additives	Concentration (g/L)
Liquid medium	Mineral water Contrex® (1L)
NH_4Cl	1
$FeSO_4$	0.005
$KHCO_3$	0.1
Na_2HPO_4	0.1
NaOH	pH 7.2

*MPN: most probable number

2.2 (Article 1) Evolution of purification efficiencies of different filter materials and impact of filtration packing height and hydraulic loading

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Abstract

Due to the overexploitation of alluvial deposits, river sands are in short supply in some areas and crushed aggregate is now being considered for use as a filter material in on-site wastewater treatment systems. This work compares the purification efficiencies of two river sands (RS1, RS2) and two crushed aggregates (CA1, CA2) using filtration reactors measuring 30cm in diameter and 30cm high over a 360-day operating cycle. Thetotal suspended solids (TSS) and organic removals display similar removal rates (TSS: 85~95%; COD: 70~90%), except for the coarse river sand (TSS, COD < 70%). The results regarding ammoniumion removals prove to be satisfactory with fine materials ($N\text{-}NH_4$: >90%). On the other hand, total nitrogen and phosphate removals are limited (Tot-N: 20%; $P\text{-}PO_4$: 33~55%) with all materials. By means of comparison, pollutant removals mainly depend on the filter material grain size. The higher total nitrogen removal rate (≈39%) is achieved by increasing the filtration bed height with fine river sand (70-cm RS1, 30-cm diameter), yet no rate increase is found with crushed aggregate (CA1: 22%). A higher hydraulic load reduces the total nitrogen removal; moreover, less stable efficiencies are noticed with TSS and organic removals. This process shows that with a deep filtration bed packed with river sand (effective size: 0.38mm) under a low hydraulic load, improved TSS, organic removals and total nitrogen removals are all achieved and remain stable over time. In contrast, crushed aggregate exhibits a better rate of $P\text{-}PO_4$ removal.

Keywords: packed bioreactor, filtration, purification, river sand, crushed aggregate, biomass.

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1. Introduction

Domestic wastewater is produced on a per-dwelling basis, in combining sewage from toilet waste with washing machine and kitchen wastewater (Reneau et *al.,* 1989; Almeida et *al.,* 1999); the characteristics of this untreated effluent are similar to those of municipal wastewater (Raunkjær et *al.,* 1994; Crites and Tchobanoglous, 1998; Palmquist and Hanaeus, 2005). In rural France, this flow rate can be as low as 180 liters / inhabitant/day. On-Site Wastewater Treatment (OWTS) plants typically comprise a septic tank for primary wastewater treatment and infiltration/filtration systems (featuring soil infiltration or sand filtration) for secondary treatment. In France, 15 million residents rely on such plants.

The septic tank enables solid/grease/liquid separation and settling, in addition to the liquefaction of solids, digestion of certain organic matter and transformation of organic nitrogen into ammonium (Reneau et *al.,* 1989). The digestion and transformation of pollutants are often incomplete, leaving high concentrations still detectable in treated effluent, known as septic effluent, which is considered to be a source of groundwater contamination and water resources eutrophication in the presence of phosphorus and nitrogenous pollutants and microorganisms (Yates, 1985; Withers et *al.,* 2011). This septic effluent is spread over soil absorption fields or sand filtration systems flowing under unsaturated conditions. This process takes place in the presence of air and water and is thus capable of efficiently removing carbon, nitrogen and suspended solids.

Secondary treatments are often applied with on-site soil absorption fields. Soil conditions however are sometimes unsuitable for wastewater treatment, hence the necessity of soil reconstruction with other packing materials, with the most widely used filter medium being natural sands from alluvial deposits, such as river sands. These packing materials must be carefully chosen in order to meet long-term operating requirements and modify purification behavior. Grain size distribution is the most important characteristic for a porous medium. French and American authorities have imposed that the particle size distribution of a filter medium meets the following requirements: an effective diameter (D_{10}) of between 0.2and 2mm, and a uniformity coefficient (D_{60}/D_{10}) of between 3 and 6 (French Standard DTU 64.1; USEPA, 2002).Some authors have also suggested a narrower range of D_{10} (i.e. $0.2mm<D_{10}<0.4mm$) so as to ensure an effective purification process (Liénard et *al.,* 2001).

The drained single-pass sand filters with discontinuous feeding are often encountered due to the simplicity of the process involved. The discontinuous feed keeps the filter medium unsaturated and aerated in addition to facilitating aerobic bacterial growth; consequently, sand filters can be considered as aerobic fixed-biomass reactors (Kristiansen, 1981a). The biomass developed inside the filter medium contributes to purification performance through the degradation of organic matter consumed by heterotrophic bacteria. The particulate organic matter is retained by the filter medium and then undergoes hydrolysis (Rodgers et *al.*, 2005). Larger particulate suspended solids are mechanically removed by straining, while the removal of smaller particles involves interception and adsorption (McDowell-Boyer et *al.*, 1986). The transformation of ammonium nitrogen into nitrates is achieved by autotrophic bacteria from the *Nitrobacteriaceae* family (Rodgers et *al.*, 2005). A number of authors have indicated that nitrification can be easily obtained in an unsaturated environment within the granular filter medium; moreover, *Nitrobacteriaceae* has been found between the surface and a depth of 12cm (Ardakani et *al.*, 1974). Nitrification often results in the high nitrate contents of treated effluent and a low total nitrogen removal rate due to a slight denitrification under aerobic conditions (Van Cuyk et *al.*, 2001). Low removal efficiencies of phosphorus pollutants (85% orthophosphates and other compounds in organic form) are prevalent with adsorption and precipitation. The adsorption of PO_4^{3-} is mainly correlated with the type of filter materials and enhanced by a greater content of clay, lime and iron and aluminum hydroxides in the materials along with a basic pH environment. Precipitation is correlated with the ionic composition of the effluent being treated: the presence of Fe^{3+}, Al^{3+}, Ca^{2+}promote the precipitation of PO_4^{3-} (Zanini et *al.*, 1998; Robertson, 2003). The elimination of microorganisms involves various mechanisms related to the cell size and activities, including straining, adsorption and predation, plus the presence of an air-water interface under unsaturated conditions (Stevik et *al.*, 2004; Chabaud et *al.*, 2006). The purification process is influenced by both environmental and operational conditions, such as: temperature, packing material characteristics, hydraulic loading, organic loading, and dosing frequencies (Ellis and Aydin, 1995; Rodgers et *al*., 2005; Gill et *al*., 2009)

Due to overexploitation, river sands are less available in some areas. Other materials are substituted for use as packing materials, e.g. crushed aggregate resulting from the quarrying of massive rocks is considered as a potentially suitable material. Some studies have shown that crushed aggregate offers a very broad size distribution with angular, irregular particle shapes (Liénard et *al*., 2001; Cho et *al*., 2006). In introducing a synthetic septic effluent, some

authors have indicated that removal efficiencies for organic and suspended solids appear to be similar for two different kinds of materials. This study however focused on short-term colonization between river sands and crushed aggregate through drawing experimental comparisons, while more long-term filter operating data were excluded (Wanko et *al.*, 2005). It was conducted using actual septic effluent in order to examine the impact of the various packing materials on filter operations, and especially on purification efficiency.

2. Materials and methods

2.1. Pilot batch experiment

The batch experiment was carried out on a laboratory-scale pilot equipped with 12 columns 30cm in diameter and 3 different heights: 15, 30, and 70 cm. The feed water was stored in a mixed storage tank and replenished about every 7 to 10 days. Figure.1 provides an overview of the pilot set-up. The feeding water was spread by the distribution disk perforated with 15 holes. These experiments were conducted in a controlled environment at 20 ± 2°C.

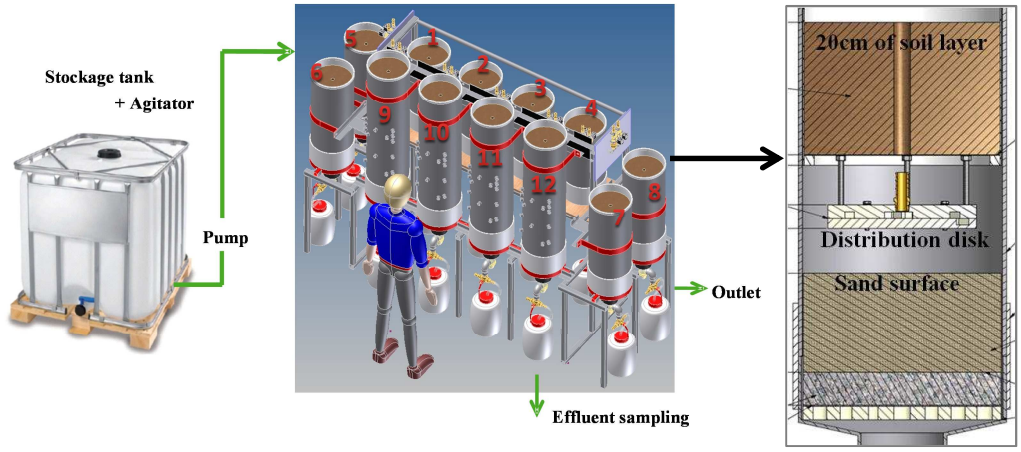


Fig.1 : Overview of the pilot batch experiment at a laboratory scale

The columns were supplied discontinuously by batches of septic effluent in order to maintain the unsaturated conditions. The hydraulic loading was set at 12cm/day for all reactors, except for columns 10 and 12, which were set at 20 cm/day.

Two natural materials (river sands RS1 and RS2) and two crushed aggregates (CA1 and CA2) were tested. The filter materials were analyzed before column packing since the river sands and two crushed aggregates were of a different type and underwent a different treatment process in the quarries. All four aggregates were studied and compared in terms of particle

size distribution, mineralogical, physical and hydrodynamic characteristics. Hydraulic conductivities were estimated by infiltration tests using the columns with an 8-cm diameter and 20-cm height (Liénard et *al*., 2001). The hydraulic residence time (HRT) was characterized in the 30- and 70-cmreactors by means of instantly injecting inert tracer lithium chloride (LiCl, 1g Li/l) and monitoring Li concentration in the treated effluents. Li was analyzed using Atomic Absorption Spectroscopy (SpectrAA 220, VARIAN).

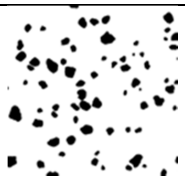
Pictures of the sand samples were processed by ImagJ Software. After an 8-bit and binary conversion, the picture scale was set and the particle analyses performed with a dedicated plugin. The filter was introduced with limitations of both size (0.1-4 mm) and circularity (>0.75) to ensure effective particle isolation. Two parameters were determined:

Circularity = $4\pi\times$ (Area) / $(\text{Perimeter})^2$;

Roundness = $4\times$ (Area)/$\pi\times$ $(\text{Major axis})^2$.

Packing material characteristics are shown in Table 1, while reactor configurations and feeding conditions are described in Table 2.

Table 1 Packing material characteristics

Filter material	River Sand 1 (RS1)	River Sand 2 (RS2)	Crushed Aggregate 1 (CA1)	Crushed Aggregate 2 (CA2)
Photograph				
Effective size D_{10} (mm)	0.38	1.60	0.17	0.44
Average diameter D_m (mm)	0.82	2.26	1.36	1.6
Uniformity coefficient (D_{60}/D_{10})	2.78	1.75	10	5
Circularity	0.849 (±0.060)	0.819 (±0.037)	0.824 (±0.088)	0.800 (±0.109)
Roundness	0.74 (±0.11)	0.76 (±0.10)	0.73 (±0.15)	0.67 (±0.15)

Fine particles (%)	0.4%	0.5%	5%	2.4%
Porosity [min, max]	[30%, 33%]	[37%, 41%]	[38%, 41%]	[38%, 44%]
Specific surface (m^2/kg)	4.04	1.18	2.78	2.88
Estimated hydraulic conductivity (m/s)	$[8.25\sim9.53]\times10^{-4}$	-	$[2.79\sim2.88]\times10^{-4}$	$[7.49\sim9.73]\times10^{-4}$
Mineralogical characteristics (mg/kg materials)				
Ca	<5	<5	795	7002
Mg	74	17	1535	11022
Na	<5	<5	295	537
K	1370	139	2356	4323
Fe	<1000	<1000	5576	32978
Al	17075	1507	38800	59383
Si	431166	460834	391779	256906

Table 2 Reactors and feeding conditions

Reactor number in Fig.1	Average height (cm)	Material	Hydraulic loading (cm/day)	Hydraulic residence time (HRT, hour)	Variation in O_2 gas
1	15	RS1	12	-	-
2	15	RS2	12	-	-
3	15	CA1	12	-	-
4	15	CA2	12	-	-
5	30	RS1	12	23	-
6	30	RS2	12	8	-
7	30	CA1	12	32	-
8	30	CA2	12	20	-
9	70	RS1	12	35	[11, 20]%
10	70	RS1	20	12	-
11	70	CA1	12	93	[19.2, 19.8]%
12	70	CA1	20	48	-

2.2. Characterization of feed water and treated effluent

The feed water was collected from the septic effluent settling tank. The main characteristics were monitored throughout the operating period. The average values of each characteristic are listed in Table 2. The treated effluents from 12 columns were characterized by physico-chemical analysis along with Merck® photometric tests for chemical oxygen demand (COD), total nitrogen (Tot-N), ammonium (NH_4^+), nitrates (NO_3^-) and orthophosphates (PO_4^3). Suspended solids were measured by weight loss after heating at 105°C for 24h. All feed water characteristics are summarized in Table 3.

Table 3 Feed water (septic effluent) characteristics

Parameter	Average value	[min; max]
pH (5 tests)	7.1	[6.6; 7.5]
TSS (mg/l, 18 tests)	39 (±11)	[20; 66]
VSS (mg/l, 1 test)	23	-
COD (mgO/l, 18 tests)	372 (±100)	[231; 572]
Tot-N (mgN/l)	81	[58; 95]
NH_4^+ (mgN/l, 22 tests)	46 (±21)	[20;77]
NO_3^- (mgN/l, 22 tests)	<2.3	[0; 2.3]
PO_4^{3-} (mgP/l, 14 tests)	9	[7.2; 12.7]
Revivifiable aerobic flora (37°C) (CFU*/100mL)	5.1×10^5	[9.5×10^4; 1.1×10^6]

* CFU=Colony Forming Unit

The total organic matter attached to the various materials was quantified as volatile dry weight (VDW), with the weight loss of sampled materials being measured by incineration between 105° and 550°C (Rolland et *al*., 2009). The total organic distribution across the depths of the four materials was evaluated at the end of the second study period.

The revivifiable aerobic heterotrophic and autotrophic flora were extracted from the three different layers in river sand 1 and crushed aggregates 1 and 2 at the end of the first period. A 20-g mass of colonized materials was sampled from 0~5cm, 10~15cm and 30cm. These materials were then washed with 100ml of a 9% NaCl solution, and 10 g of washed sample were extracted using a 20-ml phosphate tampon (0.1M at pH=8)by sonication for 15min. The suspended slurry was diluted and inoculated with nutrient agar for aerobic heterotrophic flora. The nitrifying bacteria were also assessed by a mineral liquid medium containing ammonium ions. After 3 days of incubation at 30°C, the presence of nitrites was showed by adding sulfuric acid and alpha-naphtylamine (reagent Nit 1 and Nit 2 of analytical profile index: API20E) in each cupule. In case of the absence of nitrites, zinc powder was added in order to recover the nitrates. Five copies were effectuated for each dilution.

3. Results and discussion

While practicing on-site sanitation, the packed filtration bed must contain appropriate material with a bed height of at least 70cm in order to ensure the long-term performance of the installation. As an initial step, the purification performance was compared by running the filtration reactors with a 30-cm diameter and 30-cm thickness on four different packing materials (river sands RS1 and RS2, and crushed aggregates CA1 and CA2). The upper 30-cm

filter medium is the more active part in a vertical filtration bed; also, the impact of various materials on treatment efficiencies is more significant over the upper part of the filter. Operational parameters were modified on two selected materials for subsequent comparisons; these parameters were: bed thickness and hydraulic loading. The purification efficiencies with four different filter materials were monitored in terms of particulate matter (TSS), organic matter (COD), nitrogenous pollutants (ammonium, nitrates and total nitrogen) and phosphate pollutants.

3.1. Treatment efficiencies of the filtration process with various filter materials and under different operating conditions

The filtration reactors with a 30-cm active layer were packed with four different materials (river sands RS1 and RS2, crushed aggregates CA1 and CA2) and fed with feeding water at a rate of 12 cm/day. Results obtained during 360 days of operations are presented in Figure 2.

The TSS removals (Fig. 2a) showed similar and satisfactory efficiency rates with the fine river sand (RS1) and both crushed aggregates (CA1 and CA2) after 60 days of operations. RS1 demonstrated quick and effective removals at the beginning of the process. On the other hand, the coarse river sand (RS2) yielded lower and less stable TSS removals throughout operations, typically below 80%. COD removals behaved similarly to TSS. The coarse river sand (RS2) was rated at the lowest efficiency (<70%, >125mgO/l) throughout the operating period, while the fine river sand (RS1) and crushed aggregate 1 (CA1) exhibited a similar removal rate (85%, 60mgO/l) after running for 120 days (Fig. 2b). Compared to RS1, the two crushed aggregates took longer (100 days for CA1, 180 days for CA2) to reach a similar efficiency level. While an increase in removals can be noticed in RS2, this material still produced the lowest and least stable organic removals.

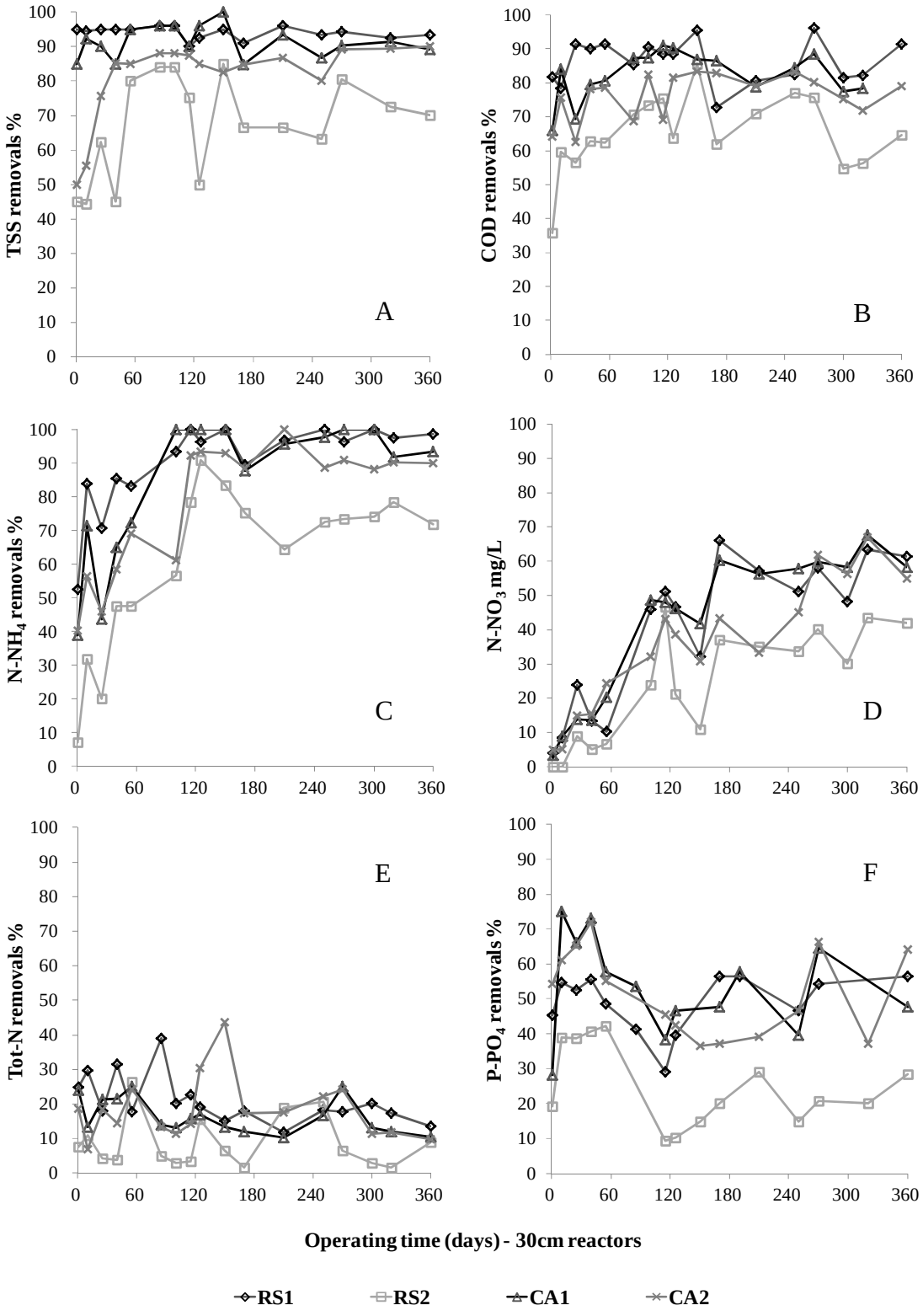


Fig.2: Treatment efficiencies of 30-cm reactors with four filter materials during 360 days of operations (a: TSS removals, in %; b: COD removals, in %; c: N-NH_4 removals, in %; d: N-NO_3 concentration, in mgN/l; e: Tot-N removals, in %; and f: P-PO_4 removals, in %)

The nitrogenous pollutants in feeding water were dominated by organic and ammonium nitrogen; nitrate traces could be neglected. As indicated in the TSS and organic removals, RS1 and CA1 also showed a similar rate of satisfactory efficiency recordings in ammonium removals after 100 days, with RS1 displaying early stabilization (Fig. 2c). The removals in CA2 indicated slightly less stability. Meanwhile, the removals with coarse sand RS2 tended to be less than 80%. The increases in nitrate concentration were observed in all treated effluents (Fig. 2d). The conversion of nitrogen forms indicate that the activities of nitrifying bacteria and nitrification taking place had been gradually optimized in the given environment (Pell and Nyberg, 1989c; Van Cuyk et *al*., 2001; Rodgers et *al*., 2005). The ammonium and nitrate results suggest significant nitrification in RS1 and CA1 and relatively less nitrification in CA2 (after 180 days), as well as an incomplete nitrification with RS2. The increases in nitrate concentration led to poor or even decreased total nitrogen removals (Fig. 2e) due to N_2 stripping after denitrification; however, denitrification remained limited due to the lack of an available carbon source and conditions favoring reduction following nitrification (Van Cuyk et *al*., 2001). The initially higher Tot-N removal rates and the non-simultaneity between ammonium removal and the appearance of nitrates suggests the possible existence of sorption or assimilation by fast-growing heterotrophic bacteria in the unstable systems packed with fine river sand and crushed aggregate (De Vries, 1972; Hinkle et *al*., 2008).

Phosphorus pollutants in the feeding water were dominated by phosphates; their removal rates were typically below 60% (2~9 mgP/l). Figure 2f shows that phosphate removals by the filtration reactors were variable and less effective. The two crushed aggregates were responsible for somewhat greater removal at the beginning of the process. Removal rates decreased after about 120 days of operations, falling in general to below 60%. The low or decreasing P-removal efficiencies have been observed by other authors as the result of an aerobic or anoxic environment; moreover, phosphorus elimination requires an anaerobic condition, which is limited in filtration beds (Gill et *al*., 2009; Torrens et *al*., 2009), and might be explained by the depletion of adsorption sites or release by the biomass (Arias et al., 2001; Vohla et *al*., 2011).

From the above discussion, RS2 presents an exceptional case due to the coarseness of this material. As the reference material, the fine river sand shows predictable and satisfactory removals in the TSS, organic matter and ammonium. The crushed aggregates, especially CA1, yield similar purification efficiencies. A further comparison of treatment efficiencies is

needed in order to differentiate the purification capacities of RS1 and CA1. The next step therefore consists of distinguishing between the reference material (RS1) and the typical crushed aggregate (CA1) during 360 days with operational alternations in bed thickness and hydraulic loading rate (HLR).

3.2. Effect of operating conditions on the crushed aggregate filter

The treatment efficiency results for river sand and crushed aggregate according to the various heights and the two hydraulic loads are summarized in Table 4.

Generally speaking, these results demonstrate, for both RS1 and CA1-packed reactors, that reducing bed thickness (from 30 to 15cm) leads to a lower purification efficiency, especially with respect to organic and ammonium removals. In contrast, the thicker beds improve efficiency and provide for both satisfactory removals and better stability due to the longer HRT (Table 2) (Torrens et *al*., 2009). With 70-cm filtration reactors, RS1 led to slightly better efficiencies in organic and ammonium removals, but also to significantly higher total nitrogen removal rates (39% for RS1 and 22% for CA1). P-removals, on the other hand, were higher in crushed aggregate (44% for RS1 and 65% for CA1).

The other comparison drawn was between HLR of 12cm/day vs. 20 cm/day with two selected filter media. Put briefly, increasing HLR led to a decrease in the HRT value, especially for RS1 (Table 2), and a slight decrease in the efficiency of several parameters (Table 4): TSS, organic matter, ammonium and total nitrogen, specifically at the beginning of operations. Removal rates gradually rose with operating time, and the bed thickness also compensated the resistance to hydraulic loads, given that some authors have indicated that a higher hydraulic loading significantly reduces organic removal efficiency in shallower beds (38cm) (Rodgers et *al*., 2005).

From the above discussion, even though RS1 and CA1 yield similar purification efficiencies, differences could still be detected during the first 120 days, particularly in the total nitrogen and phosphate removals, which presumably should be correlated with the differences in material characteristics (Fig.3). Ammonium removal efficiencies presented fluctuations under higher hydraulic loading, yet both materials seemed to be resistant enough and removals generally remained above 90%. The early stabilizations were established under the lower hydraulic loading, notably in the fine river sand (RS1), whereas a longer operating time was needed for the two materials at a higher loading. At the beginning of operations, both adsorption and incomplete nitrification were present in the systems (Wanko et *al*., 2005). The

higher water flow reduced ammonium adsorption and limited the nitrification process (Van Cuyk et *al.*, 2001; Hedström and Rastas Amofah, 2008). This impact of hydraulic loading has been confirmed by nitrate contents as well as total nitrogen removals in the higher loading reactors, where denitrification has considerably limited the reduction of HRT values. Phosphate removals were not improved under the environment of unsaturated filter media and were based mainly on particulate retention and adsorption. As mentioned in the previous section, efficiencies declined for both the two hydraulic loadings and the two types of materials.

Table 4 Treatment efficiencies of filtration reactors with two selected materials (RS1 and CA1) under different operating conditions

Operating conditions		TSS%	COD%	N-NH_4%	NO_3 (mgN/l)	Tot-N%	P-PO_4%
HLR (cm/day)	Thickness (cm)	RS1 Average value [min, max]%					
12	15cm	86 [60, 91]	71 [58, 84]	69 [6, 95]	30 [6, 55]	19 [8, 28]	33 [11, 55]
12	30cm	94 [90, 96]	86 [73, 96]	90 [53, 100]	40 [4, 66]	21 [12, 39]	50 [29, 61]
12	70cm	97 [80, 100]	96 [85, 99]	98 [74, 100]	29 [7, 57]	39 [21, 58]	44 [12, 85]
20	70cm	94 [75, 100]	92 [81, 98]	94 [67, 100]	32 [5, 47]	23 [7, 46]	57 [16, 85]
HLR (cm/day)	Thickness (cm)	CA1 Average value [min, max]%					
12	15cm	78 [45, 90]	64 [51, 80]	62 [8, 92]	31 [8, 61]	11 [2, 23]	40 [18, 57]
12	30cm	91 [84, 100]	82 [66, 91]	85 [39,100]	41 [3, 68]	16 [10, 25]	54 [28, 75]
12	70cm	96 [80, 100]	94 [80, 98]	95 [71, 100]	44 [6, 65]	22 [4, 36]	65 [33, 91]
20	70cm	90 [65, 98]	90 [81, 98]	91 [63, 100]	48 [9, 65]	17 [6, 29]	66 [47, 91]

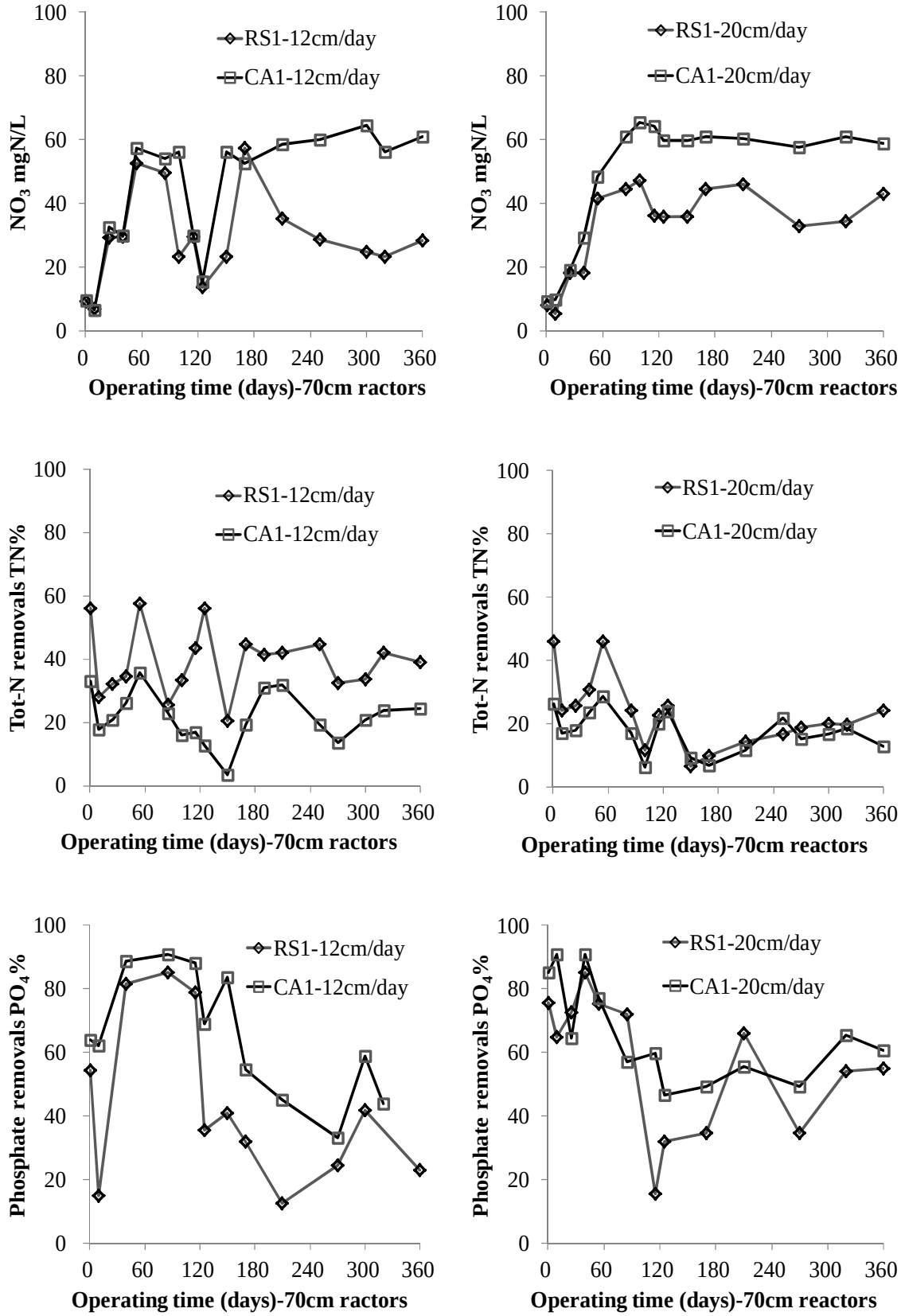


Fig.3 : Increase in nitrate ions, total nitrogen and phosphate removal efficiencies with 70-cm reactors for two materials under different hydraulic loadings

3.3. Consequences of different filter materials on filter operations: Biomass distribution and purification efficiencies

Impact of material characteristics on biomass distributions in filtration reactors

With the observations recorded for the various filter media at the end of the study, total organic contents (expressed as volatile dry weight, i.e. mgVDW/g material) and the bacterial flora of extracted cell cultures could be assessed by core sampling from 3 layers in the filtration reactors (0~5cm, 10~15cm and 30cm). Due to the poor colonization onto coarse sand (RS2), cell extractions were not carried out with this material. The results of VDW in four media are presented in Table 5, while the results of total heterotrophic flora and autotrophic (nitrifying) flora are listed in Table 6.

Table 5 Distribution of total organic matter in four materials at 360 days

Layer: VDW (mg/g aggregate)	RS1	RS2	CA1	CA2
0~5cm	12.49	3,69	10.03	8.01
10~15cm	4.10	2,19	4.81	5.21
30cm	2.98	1,63	3.39	3.56
VDW decreasing from 0~30cm (%)	76%	56%	66%	56%

Total organic contents decreased from the top layer throughout the bed in all materials studied. The fine river sand (RS1) showed higher accumulations at the surface and less organic matter in the deeper part. The total VDW is highest for RS1, in accordance with grain size (see Table 1, average grain diameter of 0.82 mm for RS1, vs. 2.66 mm for RS2). This fineness results in the surface retention of particulate matter and the straining of bacterial cells input by the feed water. In contrast, both crushed aggregate specimens revealed a slightly higher penetration of particulates and cells into the beds due to the size heterogeneity of these materials (Table 1: uniformity coefficients of 10 and 5 for CA1 and CA2, respectively, vs. 3 and 2 for RS1 and RS2, respectively).

The distribution of heterotrophic bacteria in the fine river sand appeared to be homogeneous in the top 15 cm of medium and then decreased significantly in the deeper layer, which is in agreement with the VDW values. These results indicate that in fine filter media, filtration is a surface phenomenon active over the top part of the filter, hence leading to colonization where

substrates, bacterial cells and oxygen are relatively concentrated compared to the deeper part (Pell et *al*., 1990; Ellis and Aydin, 1995). This surface filtration phenomenon is less pronounced with crushed aggregate (CA1 and CA2), despite a more heterogeneous grain size even though the top 0~5 cm still show a greater colonization of heterotrophic bacteria, although without any significant decrease from 15cm to 30cm, unlike the fine uniform river sand.

Table 6 Distributions of heterotrophic and nitrifying bacteria throughout the depth of three materials at 360 days

Heterotrophic flora (CFU*/g material)	RS1	CA1	CA2
0~5cm	4.7×10^{7} [2.5×10^{7}; 6.9×10^{7}]	4.28×10^{6} [2.2×10^{6}; 6.3×10^{6}]	2.30×10^{6} [1.2×10^{6}; 3.4×10^{6}]
10~15cm	4.98×10^{7} [2.6×10^{7}; 7.3×10^{7}]	2.98×10^{6} [1.6×10^{6}; 4.4×10^{6}]	1.25×10^{6} [6.6×10^{5}; 1.9×10^{6}]
30cm	5.05×10^{5} [2.7×10^{5}; 7.5×10^{5}]	2.93×10^{6} [1.5×10^{6}; 4.3×10^{6}]	1.66×10^{6} [8.7×10^{5}; 2.4×10^{6}]
Nitrifying flora (MPN*/g material)	RS1	CA1	CA2
0~5cm	20 [12; 48]	130 [78; 312]	50 [30; 120]
10~15cm	6.00×10^{3} [3.6×10^{3}; 1.4×10^{4}]	1.80×10^{3} [1.1×10^{3}; 4.3×10^{3}]	3.50×10^{3} [2.1×10^{3}; 8.4×10^{3}]
30cm	N/A	9.00×10^{2} [5.4×10^{2}; 2.2×10^{3}]	6.00×10^{3} [3.6×10^{3}; 1.4×10^{4}]

*CFU=colony forming unit (uncertainty: 95%, variation [min; max])

*MPN=most probable number (uncertainty: [min 40%; max 140%])

The distribution of nitrifying bacteria is less prevalent at the surface of the three filter media because the presence of higher substrate concentrations inhibits the activities of autotrophic flora. For the fine river sand (RS1), the nitrifying bacteria are mainly concentrated in the 10~15cmlayer but not in the deeper part (30cm), which suggests that the distribution of nitrifying bacteria is also influenced by another factor, namely oxygen content. The lower

oxygen gas contents (Table 2) in RS1 during batch operations indicates a relatively saturated environment and the lack of access to oxygen in the deeper layer. On the other hand, with a lower substrate, the nitrifying flora is embedded relatively deeper in crushed aggregate, due to the size heterogeneity and angularity of crushed aggregate; moreover, the porosities are relatively higher and the media less saturated (e.g. CA1, Table 2). Some authors have pointed out that nitrifying bacteria are most active in the 10-12 cm below the infiltrative surface (Ardakani et *al*., 1974; Bahgat et *al*., 1999), but with more permeable material nitrification also occurs between depths of 0.3 and 1m in the medium (Pell et *al*., 1990).

Bacteria influence the treatment efficiencies (through the final removal value and stabilization time, as described above) of soluble pollutants, especially given that heterotrophic metabolisms remove organic carbon and may cause denitrification in an anoxic environment while the nitrification metabolism removes ammonium ions.

Impact of material characteristics on the treatment performance of filtration processes:

Given that packing material characteristics govern the mechanical retention phenomena and biomass implementation, they ultimately alter the treatment performance of filtration reactors.

The removal of particulates and organic pollutants depends to a large extent on the material grain size. As observed in Figure 2 with RS1 compared to two crushed aggregates (CA1 and CA2), a medium composed of finer material provides for earlier stabilization in both TSS (particulates) and organic (particulates and dissolved forms) removals under the action of mechanical retention by the reactor surface layer (McDowell-Boyer et *al*., 1986; Rolland et *al*., 2009). Efficiency can also be improved and enhanced within relatively coarser media (CA1 and CA2), with an accumulation of retained particulates by reducing pore space and increasing surface area; the hydrolysis and assimilation by heterotrophic biomass also play an important role in organic pollutant reduction (Siegrist and Boyle, 1987; Schwager and Boller, 1997; Rodgers et *al*., 2005). Results from this study indicate that for a filter medium with an average grain size smaller than 1.6mm (i.e. D_{10}<0.44mm, CA2), 30-cm media are sufficiently effective for TSS removals (Ellis and Aydin, 1995; Elbana et *al*., 2012). Crushed aggregate exerts no significant impact on TSS and organic removals provided an adequate stabilization time has been allocated (i.e. more than 60 days of operations).

The highly permeable material (RS2) with a coarse size does not improve ammonium elimination (< 75%) due to its poor HRT (Table 2), which leads to an incomplete nitrification.

Aside from the exceptionally coarse sand, RS1 and the two crushed aggregates reveal no significant difference in ammonium removal after 100 days of operations. An earlier and faster ammonium removal by RS1 (over the first 60 days) was due to the assimilation by bacteria growth, which takes place higher in the top layer of river sand.

By drawing another comparison between RS1 and CA1 with 70-cm media, the material characteristics indicate a significant influence on total nitrogen removal efficiency. Using the 70-cm reactor packed with RS1, the improved removal (39% for RS1, 22% for CA1) is due to the more extensive denitrification within the fine medium and under a low hydraulic loading. Relatively saturated conditions (oxygen gas variation, see Table 2) when the effluent inlet arrives and stagnates at the surface of the fine uniform medium (Table 1) are temporarily created. Moreover, the pore seal caused by an accumulation of particulates and biomass further improves the appearance of local anoxic microsites and favors denitrification (Gill et *al*., 2009). The denitrification condition is fragile to a point that increasing HLR serves to limit denitrification by reducing the corresponding HRT value (23% for RS1 at 20cm/day and 39% for RS1 at 12cm/day).

Unlike with organic and nitrogenous pollutants, phosphate elimination is dictated by the physical retention of phosphate particulate sand the physico-chemical mechanisms of soluble phosphates (Clark et *al*., 1997; Molle et *al*., 2005). This difference is mainly based on the type of materials and not on the bed thickness. Crushed aggregate yields a higher phosphate removal efficiency, even though its efficiencies also decrease. The presence of a greater fine particle content and the mineralogical heterogeneity in crushed aggregate may improve the adsorption onto material surfaces and lead to temporarily immobilization inasmuch as desorption may be taking place.

4. Conclusion

This study has been based on comparisons of pollution reduction in the bed filters of On-Site Wastewater Treatment between two types of packing material, river sand and crushed aggregate, with different physical and mineralogical characteristics.

The elimination of suspended solids and organic matter is mainly governed by the size of granular materials. Effective removals (>90%) of TSS and COD were achieved by both types of materials once the material fineness was deemed appropriate. Stable removal rates were observed by increasing the filtration bed height with selected materials that possessed very

distinct characteristics. An increase in hydraulic loading slightly decreased he removal rate in a crushed aggregate (CA1), yet also resulted in a longer time before stabilization in both media.

Ammonium removals, accompanied by the enrichment of nitrate content in treated effluent, led to low total nitrogen removals. The excessive size of filter medium RS2 did not suggest a complete ammonium removal nor a complete nitrification. Longer HRT with the relatively fine media significantly improved ammonium elimination but still increased nitrate concentrations. With the deeper fine river sand medium, the nitrate level decreased after a certain operating time, which indicates denitrification is potentially more readily favored than with crushed aggregate. The exceptional loading results with a shorter HRT and the Tot-N removals decreased in both materials.

The P-removal efficiencies were relatively unstable across the tested filtration systems. The elimination rates were mainly governed by physicochemical mechanisms (adsorption onto media or organic matter and precipitation) rather than biological ones (assimilation) and moreover were not significantly affected by a hydraulic loading increase. With greater Ca contents and mineral heterogeneity, the crushed aggregates (CA1 and CA2) displayed better phosphate removal rates; however, the efficiencies also declined to under 60% after 120 days of operations.

The organic matter distributions indicate that surface retention (0~15cm) in the fine river sand and the enrichment of heterotrophic bacteria are also found in this "cake"-like layer. On the other hand, both crushed aggregates showed a deeper insertion of organic matter and attached bacteria due to their grain size heterogeneity and the angularity of their shapes. A higher nitrifying bacteria content is found at 10~15cm in the fine sand (RS1) and at 10~30cm in the crushed aggregates (CA1 and CA2).

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Conclusion:

Au-delà de la comparaison des sables de rivière avec les agrégats concassés, nous avons montré que le paramètre « taille des grains » est le plus impactant sur l'efficacité du biofiltre. L'agencement des grains de petite taille semble être le plus efficace pour arrêter les MES (matières en suspension) mais aussi en terme de colonisation (plus de MVS et plus de flore hétérotrophe). Avec une faible granulométrie, le réacteur est efficace dès une faible hauteur. Pour des grains de taille plus importante, l'abattement est augmenté pour la hauteur plus importante: de bons abattements des MES, et de la DCO (demande chimique en oxygène) ne sont observés qu'avec l'augmentation de la hauteur du réacteur. Ceci est corrélé avec le fait que le biofilm est de moindre importance en surface du réacteur mais plus présent dans les profondeurs du réacteur.

Les efficacités sont d'une manière générale liée à l'ensemble de paramètres souvent interdépendants. Il est difficile de distinguer ces facteurs si les réacteurs présentent une efficacité similaire pour l'abattement des MES et DCO, comme pour les réacteurs RS1 et CA1. Le RS1 montre un effet important de filtration en surface, ce qui implique un abattement fort en MES et DCO pour les réacteurs ayant de faibles épaisseurs (15 et 30cm). Mais le cas particulier de RS2 (grains de tailles grossières), une augmentation des efficacités avec le temps de fonctionnement (MES et DCO) observée indique une mise en place de biomasse sur la hauteur du filtre. Cependant, cette distribution longitudinale de la biomasse permet une épuration sur toute la hauteur et ainsi une épuration moins importante avec des hauteurs de 15 et 30cm qu'avec une hauteur de lit de 70cm. Les agrégats concassés, avec une distribution des tailles des grains située entre le RS1 (taille petite) et RS2 (taille grossière), doivent présenter également une colonisation en profondeur du massif filtrant, puisque les réacteurs avec CA1 et CA2 présente les capacités similaires d'abattement en MES et DCO que RS1. Les mesures des MVS et les dénombrements de la flore aérobie hétérotrophe sur des couches à différentes profondeurs permettent une mise en évidence de l'importance de la biomasse dans la profondeur par rapport à celle de la couche supérieure.

Le comportement vis-à-vis de l'azote est différent de celui des MES et de la DCO. La présence d'une phase anoxique avec un temps de séjour assez long pour qu'une partie du massif soit immergée n'est observée que pour le réacteur de 70cm en RS1 ce qui favorise la dénitrification, (environ 40% d'élimination N est réalisée).

L'élimination du phosphore peut être principalement mise en relation avec la composition minérale du matériel (phénomènes d'adsorption et de précipitation), même si une assimilation biologique par les bactéries au début du procédé peut être envisagée. L'alternance aérobie/anaérobie n'existe pas dans les conditions du réacteur filtrant insaturé ce qui est défavorable à une assimilation biologique du phosphate.

La taille des grains semble impacter plus particulièrement la colonisation des couches superficielles du biofiltre. La diminution de la biomasse en fonction de la profondeur du filtre peut être mise en relation avec le % d'humidité initiale du filtre. En effet la rétention en eau qui est observée dans les profondeurs du filtre pour les agrégats concassés expliquerait la moins forte diminution des teneurs en matière sèche volatile ainsi que celle de la flore hétérotrophe.

Il semble à l'issue de cette étude que la biomasse/biofilm soit un indicateur suffisamment sensible pour observer au cours du temps un comportement différentié du procédé en fonction des matériaux de garnissage

Par ailleurs, la présence des particules fines (en quantité plus importante pour les agrégats concassés) augmente le temps de séjour. Nous pouvons penser que les particules fines restent «coincées» dans le massif filtrant du réacteur d'autant plus que celui-ci a une hauteur importante; ce qui signifierait que l'environnement du biofiltre sera d'autant plus impacté par la présence des particules fines que la hauteur du réacteur est importante. Dans cette hypothèse ce nouvel environnement qui est différent selon la hauteur des couches et qui est généré par les particules fines pourraient impacter le biofilm et les EPS.

Chapter 3: Study of biomass development in different filter materials and evolution of biochemical compositions of total biomass and extracellular polymeric substances

Introduction :

Dans ce chapitre, il s'agit de différencier les matériaux du biofiltre (sables roulés RS1 et agrégats concassés CA1 principalement) par le profil de la biomasse (biomasse totale qui est assimilée au biofilm) ainsi qu'une partie majoritaire du biofilm, les EPS (extracellular polymeric substances). En effet comme observé au chapitre précédent, le matériel de garnissage impacte la teneur en matière sèche volatile mais aussi la quantité de flore hétérotrophe dans la couche superficielle et en profondeur du biofiltre. Les EPS sont considérées selon Flemming *et al*. (2007) comme "house of the biofilm cells". Les EPS déterminant les conditions immédiates de vie des cellules, ils peuvent être, eux aussi un indicateur pertinent pour la différenciation des matériaux dans le biofiltre.

La même méthodologie a été appliquée pour la biomasse totale et les EPS. L'évolution de la matière organique a été suivie pendant la mise en place du procédé, jusqu'à 360 jours. De plus, à l'issue de l'expérimentation, la répartition des EPS en fonction de la hauteur dans le biofiltre a aussi été réalisée.

La caractérisation de la matière organique est principalement basée sur des dosages colorimétriques des protéines, polysaccharides, substances humiques, acides nucléiques mais aussi sur la répartition en taille des matières organique assimilées à des protéines (protéine-like) et des substances humiques-like. Ces dernières caractérisations sont réalisées par chromatographie d'exclusion stérique couplée à une détection par spectrofluorescence.

Par ailleurs, au préalable nous avons voulu vérifier que l'empreinte des matières organiques de l'effluent était différente de celle de la biomasse totale ou de celle des EPS.

3.1. Experimental procedures of characterization of biomass, EPS and feeding water

The biomass developed onto the filter material is one of the main subjects in this study. It is the key to the purification performance of filtration rectors. The biomass/biofilm is characterized by its cells and EPS.

3.1.1. Sampling and total organic matters

❖ *Sampling*

During the operation period, the river sands and the crushed aggregates were sampled at different layers in the reactors from time to time. 5 sampling ports provided in the filtration reactors of 70cm height the access to the inside at following layers: 5, 10, 15, 30, 45cm, and top layer (0-2cm) could be sampled freely. About 20g materials were collected from each layer and 200g were sampled at the top layer. The accessibility to the center part of column in the depth is difficult to achieve. The extractions of biomass and EPS constituents should be carried out at once or under short term (within 24 hours) cold temperature storage (at 0-4°C). The freezing create microorganism die-off, cell lyse and release of intracellular proteins.

❖ *Dry weight and total organic matters (volatile dry weight)*

The totality of organic matters contained in sand samples is characterized by volatile dry weight (VDW). This parameter can be considered as the total biomass and expressed by mg/g dry material. About 5g material was taken from the sample, dried at the 105°C in ventilated oven during 24 hours and the dry weight is calculated as:

Equation 11: $$\mathrm{DW}\ (\%) = \frac{M_0 - M_{105°C}}{M_0} \times 100$$

The dried samples were taken from ventilated oven and calcinated in the furnace at 550°C. The weight loss between 550°C and 105°C represents the total organic matters in the sample and the rests are minerals. The results are expressed on mg/g dry material.

Equation 12: $$\mathrm{VDW} = \frac{M_{105°C} - M_{550°C}}{M_{105°C}} \times 1000$$

3.1.2. Extractions of biomass and EPS constituents

The extractions of biomass and EPS constituents from the sand medium were carried out mainly by sonication or by heating. The working conditions are summarized in Table 2.3.1:

Table 2.3.1: Experimental conditions of biomass and EPS extractions

Experimental conditions: Biomass exactions	
Extraction method: Sonication for HPSEC analysis	
Solid/liquid	30g sample / 20mL ultrapure water
Temperature	4°C
Ultrasound conditions	Ultrasound bath, 100W input (Branson, 1.9L)
Duration	60min
Extraction method: Heating for biochemical composition assessment	
Solid/liquid	10g sample / 20mL ultrapure water
Temperature	80°C
Heating conditions	Water bath
Duration	30min
Experimental conditions: EPS extractions	
Extraction method: Sonication	
Solid/liquid	30g sample / 20mL ultrapure water
Working temperature	4°C
Ultrasound conditions	Ultrasound bath, 100W input (Branson, 1.9L)
Duration	5min with 2.5min resting to avoid over heating

The extraction efficiencies were not expected to be high. It is important to realize that use of even well-standardized extraction procedure is still qualitative in nature, and perhaps only a minor part is extracted (Wingender *et al.,* 1999). The thermal treatment for biomass or EPS extraction largely interferes in the results of HPSEC fingerprints: the heat denatures the biochemical structure of macromolecules, such as proteins (Bourven *et al*, 2012). During the first time of extraction, the top layer samples were collected from the two places: side and center. The results showed in the same scales: with a difference in VDW <0.22mg/g dried material and in biochemical components sum <6mg/kg material.

After the extractions, the samples were centrifuged at 4000g for 5min in order to separate the settable materials with liquid phase, than the samples were filtrated through 0.22µm (acetate cellulose) to remove bacterial cells. The prepared samples were frozen at -20°C.

3.1.3. Biochemical compositions of Biomass and EPS

❖ *Colorimetric assays*

The quantification of extracted biomass and EPS constituents was carried out by biochemical analysis, in another word, the colorimetric assays for four major components: proteins, polysaccharides, humic substances, and nucleic acids. Some methods have been revisited in the literature review section. The methods used in this study and the analytic parameters are summarized in Table 2.3.2:

Table 2.3.2: Analytic parameters of colorimetric assays of biomass and EPS constituents

Target constituent	Method	Wavelength (nm)	Detecting range (g/L)	Standard
Proteins	Modified Lowry method	650	0.04-0.2	Bovine serum albumin
Humic-like substances	Modified Lowry method	650	0.04-0.2	Humic acid
Polysaccharides	Dubois method	492	0.02-0.1	Glucose
Nucleic acids	Burton method	600	0.005-0.05	DNA of calf thymus

❖ *3D Excitation-Emission Matrix (3D EEM) of fluorescence*

The extracted biomass and EPS samples were characterized by 3D EEM which provides the characteristics of the fluorophores. With the EEM spectra, the detection wavelengths of protein-like substances and humic-like substances could be determined for the use of SEC (steric exclusion chromatography) detection. The typical peaks and their corresponding Excitation/Emission wavelength couples have been classified by several previous studies (Figure 2.9 and Table 2.3.3):

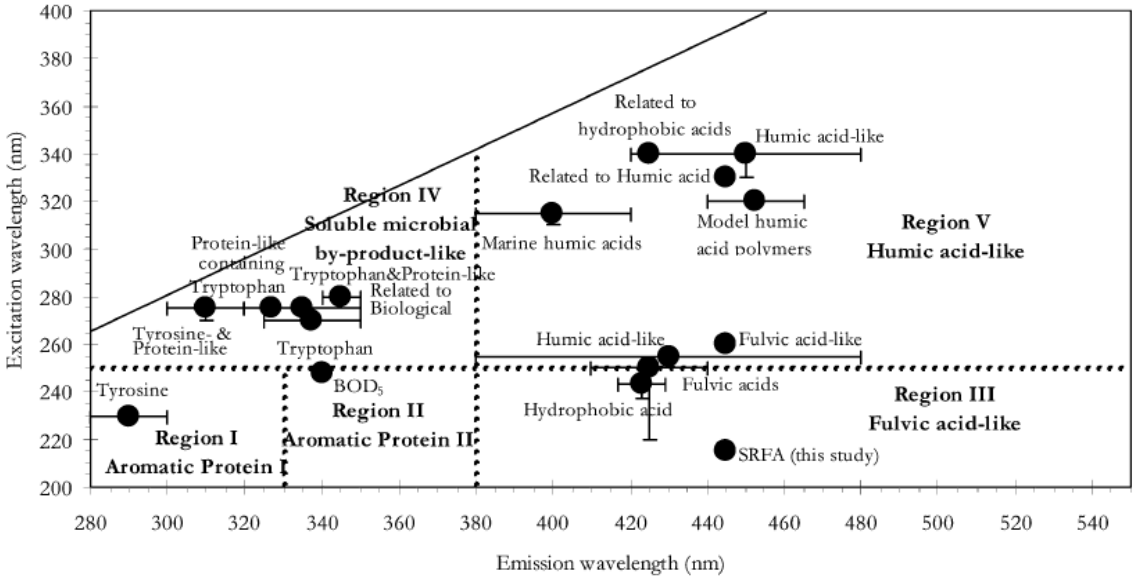

Figure 2.9: Location of EEM peaks for five EEM regions (Chen *et al.*, 2003)

Table 2.3.3: Corresponding compounds types to EEM spectra regions and Ex/Em ranges

Protein-like regions (Emission<380nm)	**Ex/Em range**	**Types of compounds**
Region I: aromatic proteins	<250nm/<330nm	Fluorescence released by protein-like compounds with the presence of tyrosine
Region II: aromatic proteins	250nm/<330nm	Fluorescence released by protein-like compounds with the presence of tryptophan
Region IV: soluble microbial by products	Excitation>250nm	Fluorescence released by components derived from proteins which contains tyrosine or tryptophan (associated or free)
Region HS-like (Emission>380nm)	Ex/Em range	Type of compounds
Region III:	Excitation<250nm	Fluorescence released by fulvic acid-like compounds
Region V:	Excitation>250nm	Fluorescence released by humic-acid-like compounds

To obtain EEM spectra, the samples were diluted with 50mM phosphate buffer at pH=7.0±0.1. The spectra were measured at 20°C, using Shimazu RF-5301 PC spectrofluorophotometer. The emission was scanned from 300 to 500nm after excitation ranged from 220 to 450nm with 15nm increments.

3.1.4. SEC coupled with fluorescence detection fingerprints

❖ *HPLC instrument and Separation*

The separations of extracted biomass and EPS were performed by High Molecular Weight (HMW) Agilent Bio SEC300Å (5-1250kDa) column followed by Low Molecular Weight (LMW) Agilent Bio SEC 100Å (0.1-100kDa) column. Samples were processed by Merck Hitachi LA Chom chromatograph equipped with a L7200 autosampler, a L7100 quaternary pump, a D7000 interface, a diode array UV detector (L7455), and a fluorescence detector (L7485).

❖ *Mobile phase*

The columns functioned with a mobile phase of 150mM NaCl and 50mM phosphate buffer (25mM Na_2HPO_4 and 25mM NaH_2PO_4) at pH=7.0 ± 0.1 with a flow rate 0.7mL/min. 100µL of filtered samples (0.2µm, acetate cellulose) was injected for each analysis.

❖ *Detection*

The UV detection was performed in the range of 210-300nm and the fingerprints were recorded at 210nm and 254nm. The protein-like fluorescence fingerprints were detected with 3 wavelength couples, Ex/Em 220nm/300nm for tyrosine-like, Ex/Em 220nm/330nm for tryptophan-like and Ex/Em 220nm/350nm also for tryptophan-like. The humic substance (HS)-like fluorescence fingerprints were recorded at Ex/Em 350nm/460nm.

❖ *Calibration*

The apparent MW for association of columns was calibrated using six proteins or amino acids with MW values of 440000, 155000, 69323, 5777, 362 and 181 Da (ferritine (Sigma), immunoglobulin G from human serum (Sigma), albumin from bovine serum (Sigma), insulin from porcine pancreas (Fluka), thyrotropin-releasing hormone (Fluka), and tyrosine (Fluka),

respectively). For mass calibration curves, the logarithm of the molecular mass (log (MW)) is plotted as a function of the elution volume (mL).

Log (MW) = -0.3164 Ve (mL) 9.4676 (R2= 0.982)

With MW: molecular weight in Da and Ve: elution volume in mL. The permeation volume determined with $NaN_3$is 22mL.

The chromatograms are established of the fluorescence intensity (volts) and the elution volume (mL). Several fractions were identified as the interval of elution volume (ex: 12~18mL). The calculation of fraction area percentage is based on the ratio between the chromatogram area of one fraction and the total area of chromatogram. The calculation was accomplished by the software Origin 6.0. The fraction area percentage is defined as:

F% = 100×Area of fraction/Total area of chromatogram

3.2. (Article 2): Evolution of biochemical compositions of biomass developed in different aggregates for the use of filter materials of onsite wastewater treatment systems

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Abstract

Due to the overuse of alluvial deposits, river sands are lacking in a number of areas, leaving crushed aggregate as a candidate filter material for onsite wastewater treatment systems. This study compares the evolution of the biochemical composition of biomass developed in river sand and crushed aggregate over 360 days of colonization by means of conducting batch experiments with septic effluent. By drawing comparisons with the biochemical composition of septic effluent, the results herein show an increase with operating time in the protein fraction of organic matter accumulating in the top layer: the protein/polysaccharide ratio rises from approx. 1 to 2.44 for river sand and to 1.98 or 3.37 for crushed aggregate. The HPSEC protein-like fingerprints show a similar trend across the three aggregates with operating time: enrichment of the high molecular weight protein-like fraction (>1,000kDa). This fractional percentage however is clearly higher for crushed sand after 210 days of colonization, and the percentage of the 6kDa-1,000kDa fraction still increases over time for a crushed aggregate, whereas for river sand it sharply decreases. Moreover, the quantities of biomass and biochemical components indicate a delayed stabilization in the presence of crushed aggregates due to their different physical characteristics, e.g. size, porosity.

Keywords: packed bed bioreactors; filtration; biomass; protein; river sand; crushed aggregate

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1. Introduction

Onsite wastewater treatment (OWTS) systems affect the health of local residents and their living environments in decentralized areas throughout the world. Household wastewater contains high concentrations of pollutants with an organic discharge of about 77g BOD/day per capita [1]. Common configurations involve septic tanks for use as pre-treatment, with the application of natural or reconstructed secondary treatment, e.g. soil adsorption fields or trenches, constructed wetlands or sand filters [2-4]. Sand filtration has become more popular for treating domestic wastewater in light of its efficiency, ease of maintenance and limited energy costs. This application requires suitable materials, such as river sands. However, natural deposits have been more heavily exploited, to a point of even being lacking in some areas, hence requiring the search for similar materials, such as crushed aggregate. The question then gets raised over the risks and consequences of altering packing materials.

Sand filters function like a fixed, porous biomass reactor for the medium that includes mechanical retention acting through the sand grain network with biological degradations due to the biomass developing around these grains. Suspended solids are physically removed by straining [5]. The retained organic carbon pollutants undergo hydrolysis and biologically degrade under the aerobic reactions of heterotrophic bacteria [6, 7].

This filter enables a gradual process of pore space reduction, in comparison with the initial state of clean beds, which is introduced by the accumulation of suspended solids, precipitates and the colonization of biomass [8-10]. The non-cellular particulates may originate from both the feed effluent and fine mineral particles dissociated from the packing materials. The biomass system reaches a steady state whereby biomass growth and organic abatement occur after a given operating period [11]. The biomass is produced by cells attached to the filter medium and plays an important role as biodegradation agent. The biomass also contributes to physically changing the porous medium [12-14]; it is a complex spatial network of organic matter composed mainly of bacterial cells. For the most part, its constituents are proteins, sugars, lipids and nucleic acids, i.e. extracellular molecules produced by cells (in the case of proteins and sugars) and captured molecules such as humic-like substances, minerals [15-17]. When a non-clogging condition is active, the non-polysaccharide fractions (e.g. proteins, humic-like substances, lipids) are found in greater quantities than polysaccharides in the biomass [18]. Protein is a component of all living cells [19]. Some of these extracellular molecules act as "cement" that binds other particles and potentially causes “pore sealing"

[20]. The proteins serve important functions in the biomass, namely structural (capsular peptides) and enzymatic (biodegradation) [21, 22].

In considering the new materials for sand filter packing, studies conducted have shown that crushed aggregate must be widely graded in size, rather than being heterogeneous in form and surface, and more varied in mineral composition than natural aggregates [23-25]. The authors also suggest that the biomass growth rate would tend to be slightly lower for crushed aggregate compared to river sand of a similar size during the feeding period [26]. The roughness of the medium surface also influences biomass maintenance [27]. The characteristics of crushed aggregate can modify the hydrodynamics and oxygen distribution relative to natural sands, and these differences may lead to a difference in filter operations.

In this study, two types of packing materials will be studied and compared: natural sand stemming from alluvial deposits (Loire River sand), set as the reference material; and two crushed aggregates resulting from quarry production. This work will seek to determine the impact of packing materials on biomass development throughout the operating period, in terms of quantity and especially the evolution in main biochemical components.

2. Materials and methods

2.1. Batch experiments (cylindrical filtration reactors)

Filtration reactors 70cm high and 30 cm in diameter with sampling ports were packed with river sand and crushed aggregate, respectively (see Fig. 1). These reactors were fed with septic effluent that had been stored in a mixed tank and replenished every 7 days. The reactors were fed discontinuously, at a hydraulic loading rate of 12cm/day in 10 daily batches.

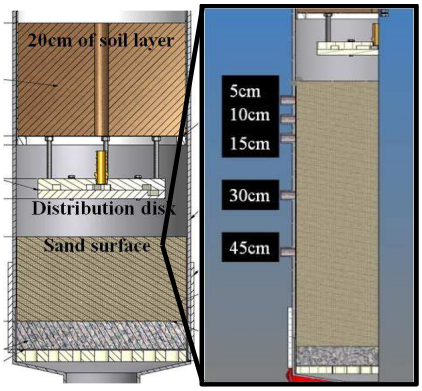

Fig.1. Filtration reactor with sampling ports

2.2. Packing material characteristics

The filter materials were analyzed before column packing since the river sand and two crushed aggregates are of different compositions and involve different treatment processes in the quarries. The three aggregates were studied and compared in terms of particle distribution, mineralogical, physical and hydrodynamic characteristics.

The particle size distribution analysis was conducted by means of mechanical shaking with a series of sieves with the following opening diameters: 0.08, 0.16, 0.2, 0.25, 0.315, 0.4, 0.5, 0.63, 0.8, 1, 1.6, 2, 2.5, 4, and 5mm. The fine particle content is defined as the percentage of the sample with diameters less than 80μm.

A water retention analysis was performed using columns 8cm in diameter and 70cm high, fed with clear water at a flow rate of 10mL/min until reaching steady state. The ratio of the retained water volume to the material volume was set as the volumetric retention capacity (%). After draining the columns, the weight loss at 105°C was measured with the wet filter materials, and the ratio of weight loss to packing material weight in the column was evaluated as the mass retention capacity of static water (%). Hydraulic conductivities were estimated by infiltration tests using the columns 8cm in diameter and 20cm high [23]. The hydraulic residence time (HRT) was measured via instant injections with lithium chloride solution(1g Li/L), and the lithium concentrations were identified by atomic absorption spectroscopy (SpectrAA 220, VARIAN). These characteristics are listed in Table 1.

2.3. Feed water: Septic effluent characteristics

The feed water was collected from the septic effluent settling tank, with all main characteristics being monitored throughout the operating period. The average and extreme values of each characteristic are reported in Table 2.

2.4. Biomass extraction

Three samplings were carried out during some 12 months of operations. The samples were collected from 5 different depths inside the reactors (5, 10, 15, 30 and 45cm) and from the top layer (0-2 cm). The total organic matter contents of each layer were assessed by the volatile dry weight (VDW, in mg/g of material). The biomass was extracted as quickly as possible (<24h) by heating to 80°C during 30min for the colorimetric analysis and by sonication at 4°C during 60min for the size exclusion chromatography (SEC) fingerprint analysis [28, 29]. The SEC fingerprint analysis actually reveals the evolution of molecular weight (MW) and how a heat treatment should modify the MW fingerprints [30].

Table 1. Characteristics of river sand (RS1) and crushed aggregate (CA1) as main packing materials, with CA2 providing a supplementary material

Material	River sand (Reference: RS1)	Crushed aggregate (CA1)	Crushed aggregate (supplemental: CA2)
Particle size distribution characteristics			
Effective size D10 (mm)	0.38	0.17	0.44
Average diameter D_m (mm)	0.82	1.36	1.60
Uniformity coefficient (D60/D10)	2.8	10.0	5.0
Fine particles% (<0.08mm %)	0.4%	5.0%	2.4%
Physical and chemical characteristics (average values; 5 tests)			
Real density (kg/m^3)	2525 (±42)	2438 (±111)	2546 (±51)
Porosity (%) [min; max]	[30%; 33%] (±1.5%)	[38%; 41%] (±1.7%)	[38%; 44%](±3%)
Specific surface (m^2/kg)	4.04	2.78	2.88
Hydraulic characteristics (average values; 5 tests)			
Estimated hydraulic conductivity (m/s)	[8.25~9.53] ×10^{-4}	[2.79~2.88]×10^{-4}	[7.49~9.73] ×10^{-4}
Water retention capacity after drainage (static water %)	8.4%	12.7%	10.0%
Hydraulic Residence Time (hours) (1 test)	35	93	-
Variation of O_2 gas level at 10cm [min, max%]	[11, 20]%	[19.2, 19.8]%	-

Table 2. Feed water characteristics

Parameter	**Average value**	**[min; max]**
pH (5 tests)	7.1	[6.6; 7.5]
TSS (mg/L, 18 tests)	39 (±11)	[20; 66]
VSS (mg/L, 1 test)	23	-
COD (mgO/L, 18 tests)	372 (±100)	[231; 572]
Revivifiable aerobic flora (37°C) (CFU*/100mL)	5.1×10^5	[9.5×10^4; 1.1×10^6]

CFU*=Colony-forming unit

2.5. Biomass characterization

Total organic contents were determined by the weight loss of sampled materials, as measured by incineration between 105°C and 550°C [31].

The biochemical components of biomass were quantified using colorimetric methods: proteins and humic substances by the modified Lowry method in introducing Folin's reagent [32]; polysaccharides by Dubois' method; and nucleic acids by Burton's method [33, 34].

The MW distributions of biomass proteins and humic substances were analyzed by HPSEC (Merck Hitachi LA Chrom Chromatograph) coupled with fluorescence detection. The high MW separation was performed by the Agilent column, bioSec, 300A (5-1250kDa) and the low MW separation by the Agilent column, bioSec, 100A (0.1-100kDa). All columns were placed in series for the separation improvement step [35]. The mobile phase was composed of 150mM NaCl and 50mM phosphate buffer at pH 7.0. The apparent MW for associating the columns was calibrated using six proteins or amino acids with MW values of: 440000, 155000, 69323, 5777, 362 and 181 Da (respectively ferritine (Sigma), immunoglobulin G from human serum (Sigma), albumin from bovine serum (Sigma), insulin from porcine pancreas (Fluka), thyrotropin-releasing hormone (Fluka), and tyrosine (Fluka)). For mass calibration curves, the logarithm of the molecular mass (log (MW)) is plotted as a function of the elution volume (mL), i.e.:

$$\text{Log (MW)} = -0.3164\ Ve\ (mL) + 9.4676\ (R2 = 0.982)$$

With: MW: molecular weight (in Da); and Ve: elution volume (in mL). The permeation volume determined with NaN_3 is 22mL. The Ex/Em wavelength fluorescence detection for protein tryptophan-like substances was found to be 222nm/330nm, while for humic-like substances it was 350nm/460nm. These wavelengths were evaluated after a three-dimensional spectrofluorimetric analysis [36].

The chromatograms are established from both the fluorescent intensity (volts) and elution volume (mL). Several fractions were identified as the interval of elution volume (e.g. 12~18mL). The fraction area percentage calculation is based on the ratio of the chromatogram area of one fraction to the total chromatogram area. This calculation was achieved using the software package Origin 6.0. The fraction area percentage is defined as follows:

$$F\% = 100 \times \text{Area of fraction/Total area of chromatogram}$$

3. Results and discussion

3.1. Treatment efficiency with two types of materials

The filter medium physically removed the suspended solids retained them within the medium. Organic carbon pollutants are removed biologically through the heterotrophic bacterial uptake. Removal efficiency results on both TSS and COD are shown in Figure 2.

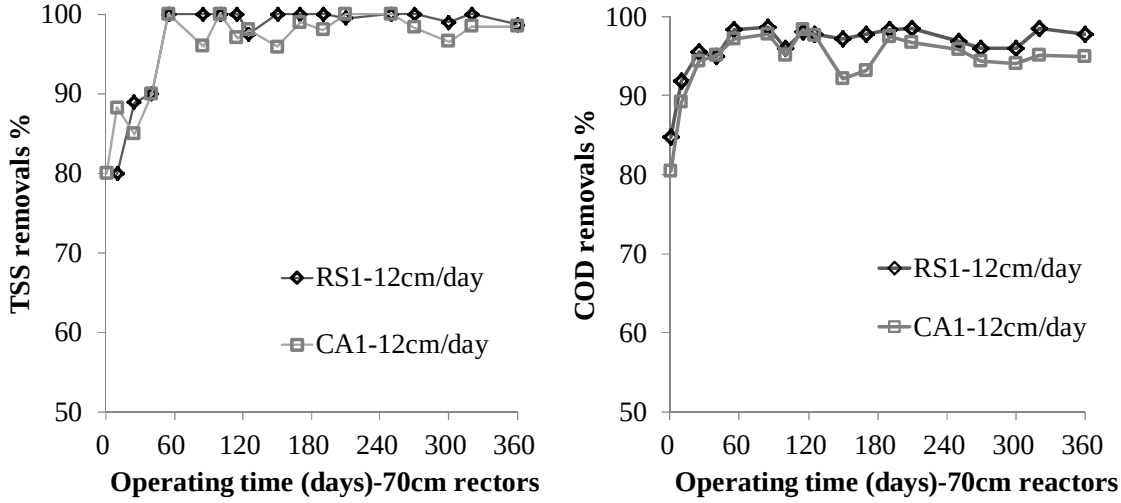

Fig.2. TSS and COD removal efficiencies with river sand (RS1) and crushed aggregate (CA1) in 70-cm filtration reactors

The suspended solid removals stabilized after some 60 days of colonization for both river sand (RS) and crushed aggregate (CA1). Variations were observed due to the feed water, though all removals typically exceeded 95%. The organic (COD) removals also stabilized after 60 days of operations, although the river sand demonstrated more stable efficiencies than CA1. The COD abatements by river sand were generally between 95%and 98%, while crushed aggregates showed similar efficiencies albeit with greater variations (92% ~ 98%). Similar trends were observed with the TSS removals. Both filter media were sufficiently effective for suspended solids and organic pollutant removals; moreover, the efficiencies were in line with previous studies [3, 6, 7, 37]. The TSS removals were primarily governed by a mechanical filtration process. During the first month, the reactor with river sand showed quicker improvement of TSS removals. After 1 month of colonization, efficiency was optimized due to grain size fineness and regularity (see Table 1). The authors also pointed out that grain sizes do exert a great impact on suspended solids removal [38]. For both of these materials, the most noteworthy change took place around 8 weeks (50-60 days in Fig.2) when comparing the performance before and after. This early behavior was assumed to be due to

TSS filtration, which caused pore blockage and increased the filtration area being utilized [6, 39]. COD removals by two materials indicated this improvement with respect to TSS removals. The elimination of organic pollutants depended on both the hydraulic residence time and development of a heterotrophic biomass. For starters, CA1 displayed higher HRT (93hours) than river sand (35hours); this observation may be explained by the clayey fine particles in crushed aggregate that tend to retain the solutes, which was not observed in river sand. The river sand, on the other hand, showed rapid abatement above 90% after roughly 10 days of operations; this finding may be due to the effect of filtration. The significant improvement in COD removals at around 8 weeks of colonization in both media could be ascribed to biomass growth, especially in the surface layer (0~2cm), where the retentions of both bacterial cells and the substrate were greatest [7, 40]. Accordingly, the organic contents inside several filter layers revealed increases for the two media, both of which were assessed by VDW during operations (Fig. 3).

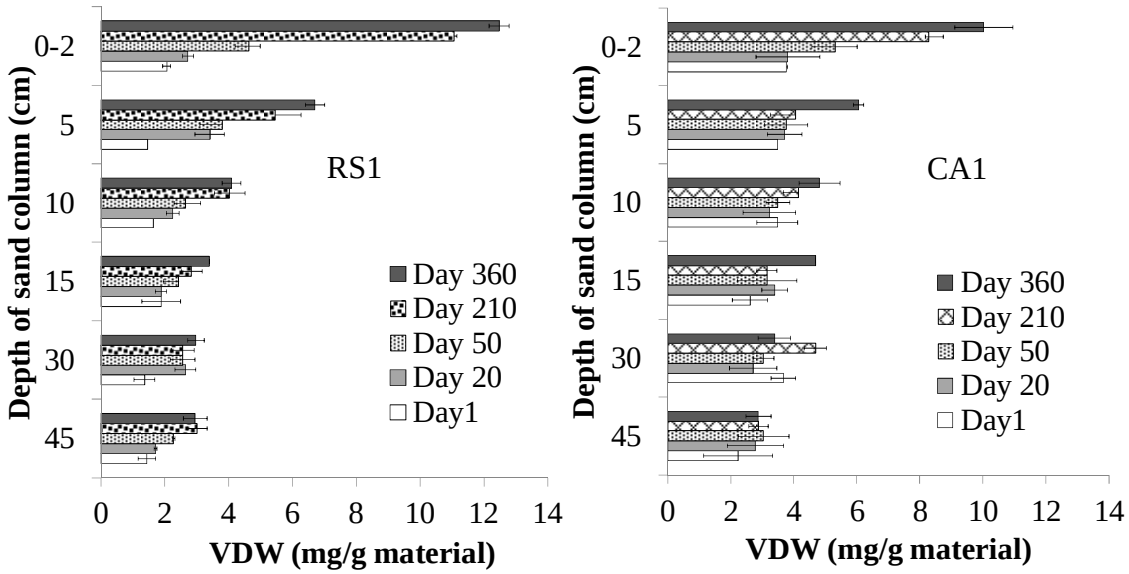

Fig.3. VDW distributions along the depth of two types of filter media, i.e. river sand (RS1) and crushed aggregate(CA1), over the entire operating period

Throughout the operating period, the accumulations of organic matter increased in all sampling layers for both media. Higher VDW in the 0~5cm surface layers of both filter media reflected higher organic accumulations compared to the VDW in deeper layers. The river sand has shown relatively greater retention of organic matter at the reactor surface, in accordance with grain size [13]. Except for the higher accumulations on the surface layer, the VDW distributions below 10cm were quite homogeneous with respect to depth. Zhao et *al*. and Lloréns et *al*. have made similar observations on both the laboratory and field scales, namely

the accumulations of organic matter were more pronounced at the surface, especially in the presence of particulate matter [14, 41]. At the beginning of operations, CA1 showed higher organic accumulations with depth, unlike the river sand. This finding might also be related to the grain size heterogeneity and higher porosity of CA1, which allows particulates or dissolved organic substances and bacterial cells to reach the deeper layers [37]. Over the next 50 days, the river sand increased more substantially in every layer than did the CA1, which could be due to the colonization of bacterial cells and excretion of extracellular polymers. Small increases were found from 210 to 360 days in the river sand across all layers. On the other hand, the VDW in CA1kept increasing after 210 days, especially in the top 0~10cm.On the higher parts of the reactors, organic accumulation included matter from the feed water and biomass growth within the filter media. At the same time, the increases in organic degradation efficiencies for both materials indicated the presence of biological activity in heterotrophic bacteria under aerobic conditions [13]. With a focus on accumulated organic matter, the evolution of biochemical fractions of top layers in two filter media was studied from time to time with respect to feed water characteristics.

3.2. Influence of feed water filtration on organic matter accumulation

As evidenced by the VDW value, organic accumulation was more abundant in the top 0~5cm. The biochemical fractions of extract from solid or liquid samples were quantified by employing colorimetric methods. The results from river sand extracts were compared to the feed water (septic effluent), as displayed in Figure 4. The proportions of these components vs. time are shown in Figure 5.

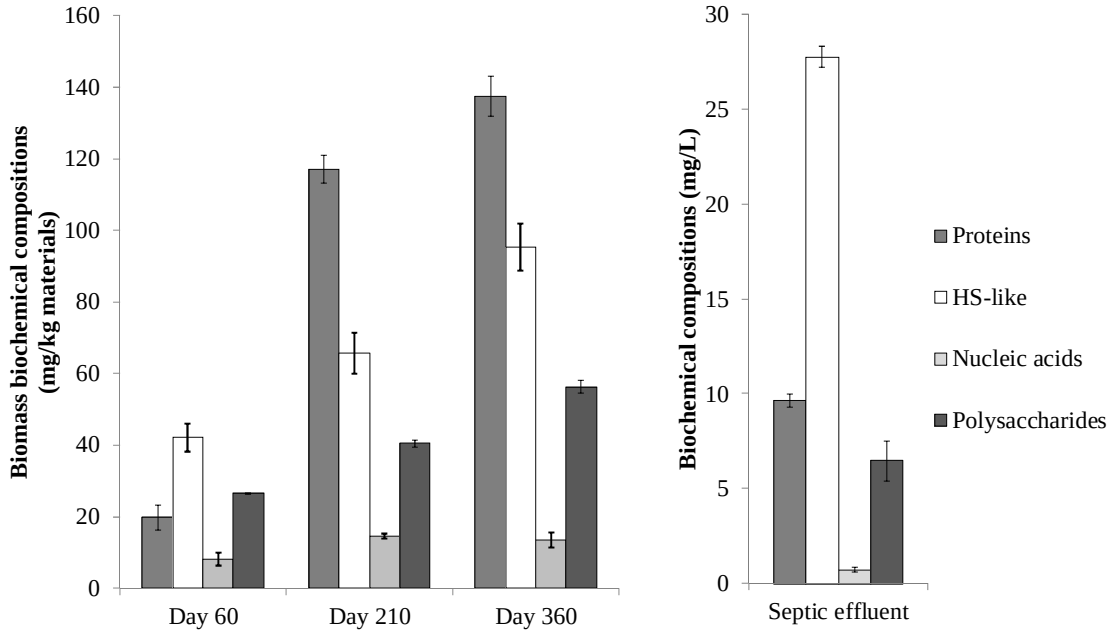

Fig.4. Biochemical compositions of biomass: (left) in the top layer of river sand throughout the operating period; and (right) of the septic effluent as feed water

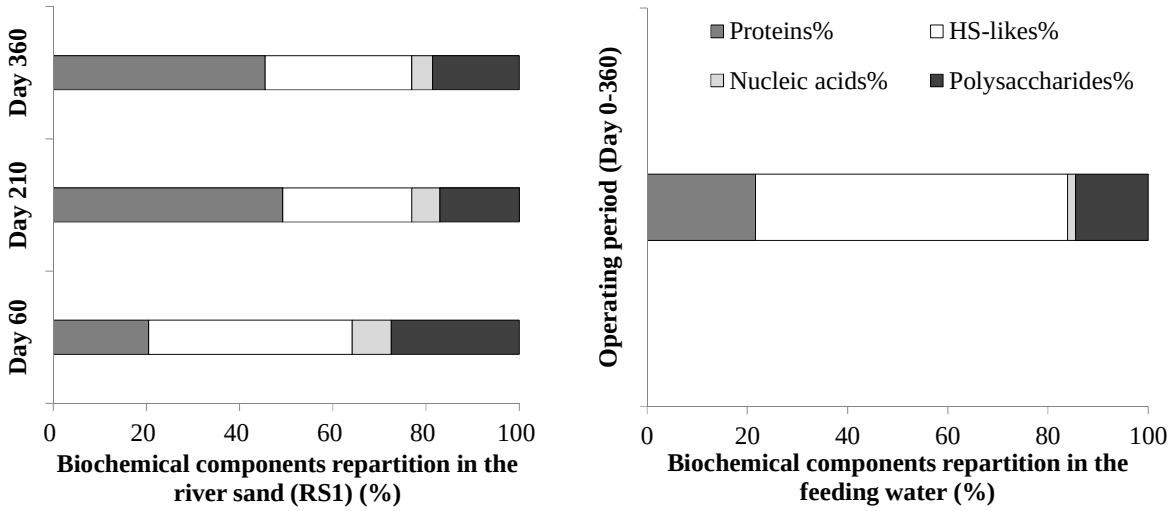

Fig.5. Repartitions of biochemical compositions of the biomass in river sand (RS1) throughout the operating period and in the feed water

In considering that the biochemical compositions in feed water remained relatively constant, Figures 4 and 5 track the evolution of the biochemical compositions of sand extracts over the operating period. All major components, i.e. proteins, humic-like substances (HS-like) and polysaccharides, increased in quantity terms and the nucleic acids achieved stability after 210 days. In comparison with the feed water, the first extract around 60 days mainly reflected the

retention of organic matter from the influent since at the time; the biomass was of a relatively small quantity. HS-like dominated the biochemical composition of the river sand extracts, which were assumed to have been derived from the re-polymerization of biologically or chemically-degraded environmental organic matter [42]. During the second extraction time (210 days) however, the biomass evolved: an increase in proteins as well as nucleic acids indicated that colonization by biomass had become a dominant origin. The 360-day extraction showed slight increases in proteins, HS-like and polysaccharides. The ratio between proteins and polysaccharides (PN/PS) rose from 0.75 to 2.44 vs. time and remained nearly constant between 210 and 360 days of operating time. This increase in PN/PS ratio is considered an indication of biomass strength; for ratio values around 1, the biomass is fragile and loose [43-45]. Figure 5 shows that the proteins and HS-like represent respectively about 45% (±5%) of the major compositions for the 210 and 360-day extractions, while the polysaccharide fractions decreased from 30% to less than 20%. This result was in agreement with several sewer biomass studies suggesting that proteins and HS-like were in fact the predominant components in natural biomass [15, 18, 46]. Given that the HS-like are environmentally generated, the evolution in protein-like compounds was considered as a present indicator of biomass: the PN/HS ratio increased from 0.47 to 1.44 until 360 days of colonization [42]. A further qualitative assessment of MW distributions by HPSEC fingerprints between the organic matter of the river sand extraction and the feed water could thus be described.

Figure 6 shows the protein-like SEC fingerprints of biomass from river sand extractions at 60 and 210 days and of the feed water. In comparing the results of 60-day biomass extraction and the feed water organic composition, certain peak locations in the protein-like fingerprints overlapped, despite their peak areas not being the same. Four groups of peaks or four MW fractions can be distinguished on the chromatograms displayed in Figure 6.

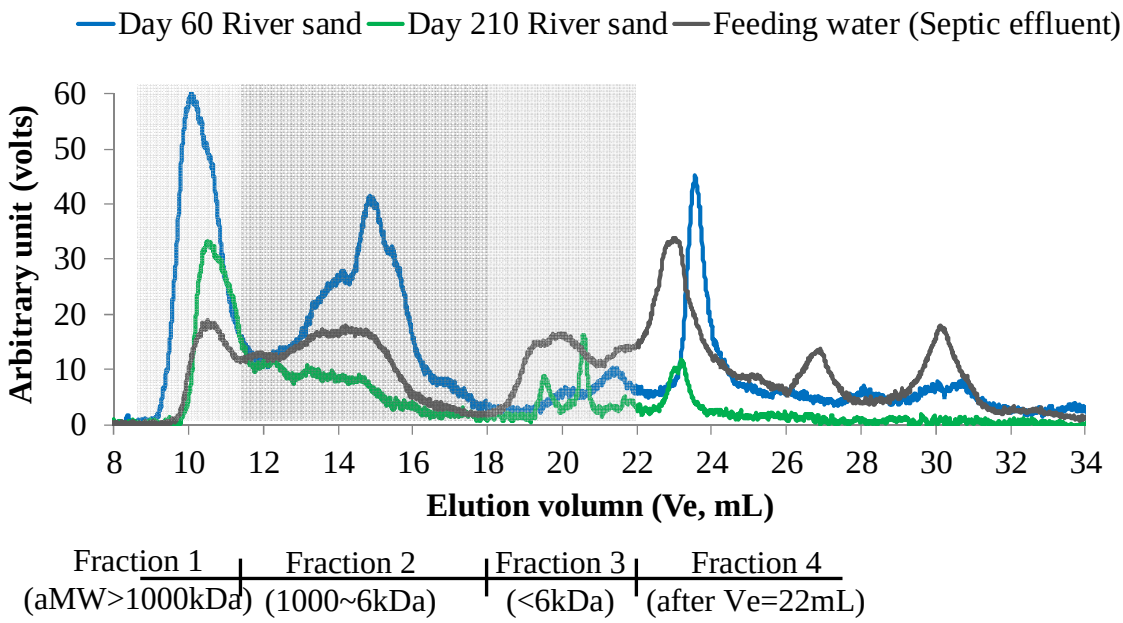

Fig.6. HPSEC protein-tryptophan-like fingerprints of the biomass extracted from river sand after 60 and 210 days of operations and of the feed water (septic effluent)

Fraction 1 (Ve < 11.75mL): one earlier peak with an apparent MW (aMW) ≥ 1000kDa;

Fraction 2 (11.75mL < Ve < 18mL): clusters of peaks not well isolated and often with two peaks shown (1000kDa > aMW > 6kDa);

Fraction 3 (18mL< Ve < 22mL): well isolated clusters of peaks (aMW < 6kDa);

Fraction 4(Ve > 22mL): one or more peaks depending on the sample. For an elution volume >22mL, the molecules are eluted beyond the total permeation volume of the column.

The % area of SEC fingerprints (fractions 1 through 4) at 60 and 210 days of river sand extractions and septic effluent were calculated and presented in Figure 7.

The comparison between septic effluent and river sand biomass clearly reveals the differences and evolution of protein-like molecular weights. The septic effluent contains a high proportion of protein-like molecules of low MW (Fraction 4 > 45%), compared with the fractions of higher MW, thus indicating the existence of heavily degraded compounds. This high MW fraction (Fraction 1 ≥ 1000kDa) represented just 10% of the total area fractions, and this percentage was lower than Fraction 2. The extraction at Day 60 from river sand showed a different pattern than that of the septic effluent: an increase in area fractions (1 and 2) with

high MW protein-like compounds, plus a similarity with septic effluent, i.e. Fraction1 (%) < Fraction 2 (%). With the extraction at Day 210, the fraction of very high MW (Fraction 1) represented over 35% of the total sample; Fraction 2 tended to stabilize at a level below that of Fraction 1. Compared to feed water, the evolution in chromatographic fractions suggests that these extracted protein-like molecules of a biomass of a system that has not reached stationary phase (60 days) might be polymers, derived by bacterial synthesis (Fraction 1) or partially conveyed by septic effluent, retained by the top layer with a similar level (Fraction 2). The increase in Fraction 1 compared to Fraction 2 suggests change in molecular configuration during the biomass maturation process (from 60 to 210 days). This configuration change could be detected due to either the analytical method or the actual presence in the biomass of very high MW compounds. Such compounds could be molecular associations, e.g. polymers found in an extracellular matrix in the biofilm of the biomass [35].

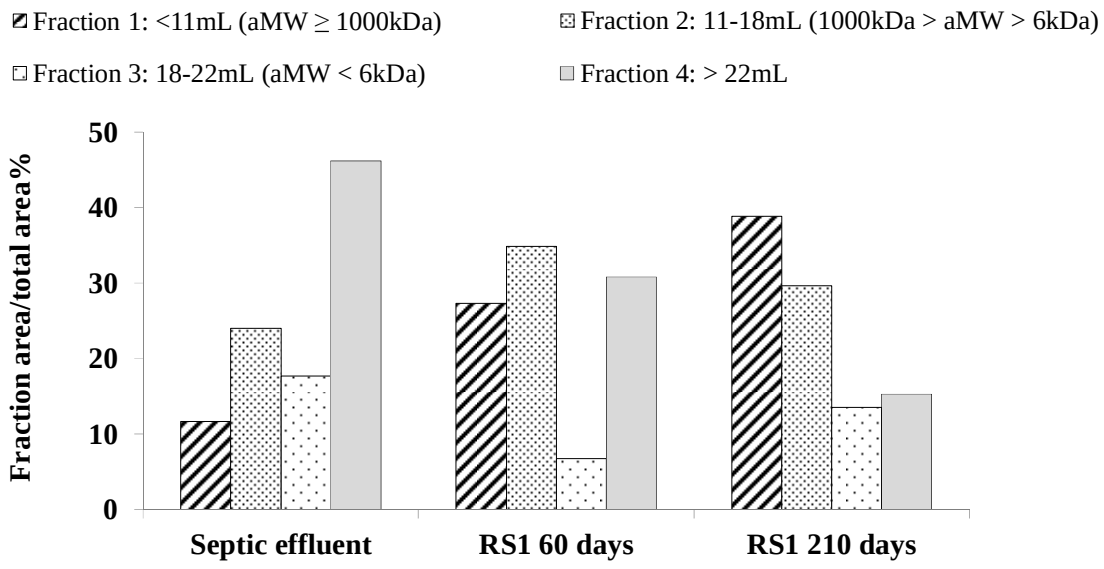

Fig.7. Evolution in the fractions of major group areas relative to total chromatographic areas of septic effluent and river sand biomass extraction at 60 and 210 days

Based on this observation, the biomass in two crushed aggregates was also assessed by comparison to the reference material: river sand, using CA1 as the primary material; and CA2 as a supplemental input (S1, S2). Their respective characteristics are summarized in Table 1.

3.3. Impact of filter medium on the qualitative characteristics of biomass

As indicated by the total organic contents (VDW) in Section 3.1, the CA1 showed only slightly lower quantities at the top layer, yet these remained of the same scale both after 210 days of colonization (11mg/g RS1vs. 8mg/g CA1) and at the end of the process (13 mg/g RS1 vs. 10mg/g CA1). The CA2 exhibited a slightly lower final VDW (8mg/g aggregates, see supplemental data, S1). A more specific analysis was needed to investigate the impact of materials with different characteristics on biomass quality. The evolution of biochemical compositions in CA1 was also compared to the reference material. These results (using river sand as the reference) are displayed in Figures 8 and 9.

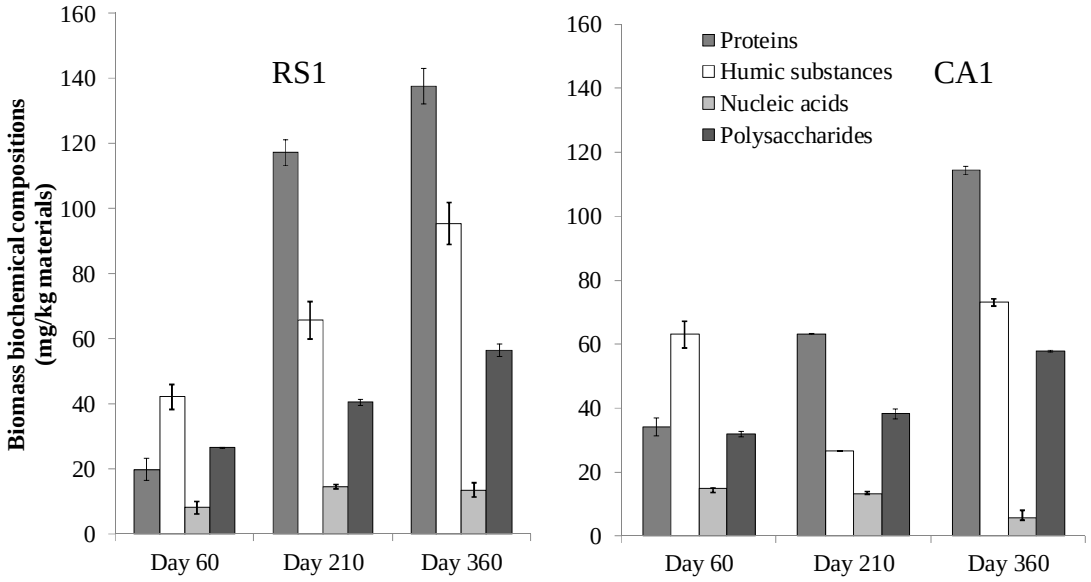

Fig.8. Biochemical compositions of biomass extracts of both river sand (RS1) and crushed aggregate (CA1)

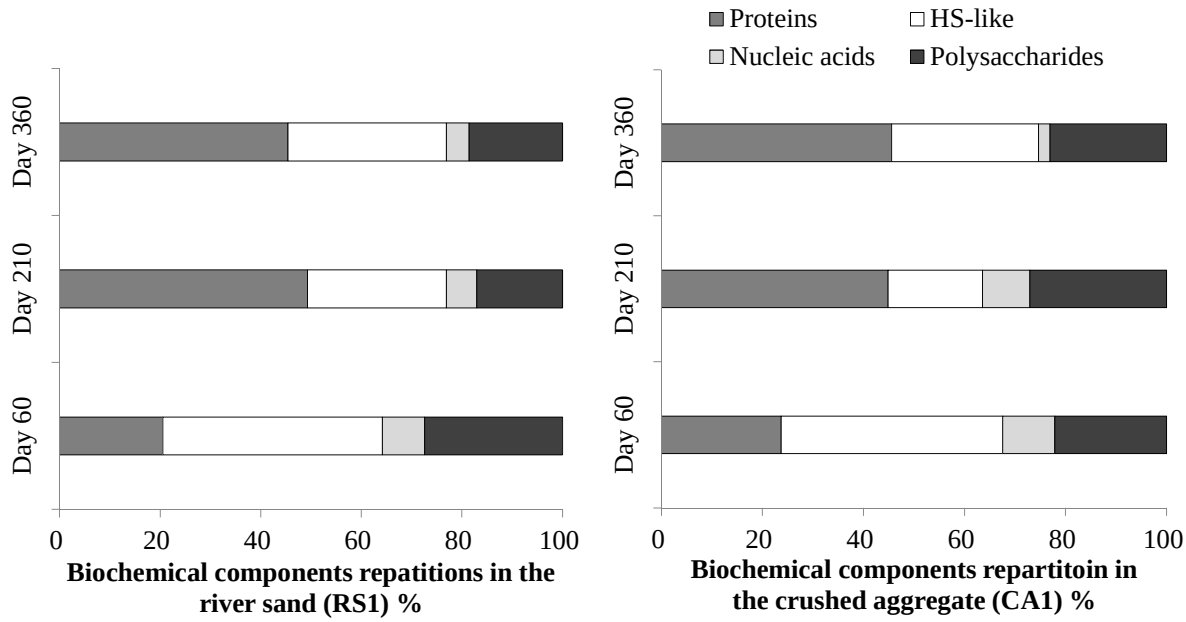

Fig.9. Proportions of biochemical components extracted from river sand (RS1) and crushed aggregate (CA1)

The biomass extracted from CA1 showed a similar organic retention behavior at the beginning of the process. Like for the river sand, increases in protein fraction have been observed, and the trend in biochemical fraction evolution did not deviate significantly (Fig. 9). In accordance with the VDW, the biochemical compositions of CA1 (Fig. 7) yielded smaller quantities than RS1 with all components except for polysaccharides. The PN/PS ratio increased from 1.07 to 1.98 (river sand: 0.75~2.44), while the PN/HS ratio moved from 0.54 to 1.57 (river sand: 0.47~1.44). The CA2 (see supplemental data, S2) provided a similar percentage change (PN/PS: from 0.77 to 3.37; PN/HAS: 0.24 to 1.22), yet with even lower quantities of biochemical components. The similar trend in biochemical fraction evolution suggests that the biomass growth mechanism undergoes the same process as in river sand, despite the crushed aggregate characteristics differing significantly.

However, the quantities of biochemical components showed a biomass stabilization in river sand from 210 to 360 days (e.g. proteins only rose from 120 to 140 mg/kg) that was not replicated in CA1. The latter exhibited much lower components around 210 days and then significantly increased around 360 days (proteins jumping from 60 to 120mg/kg, see supplemental data, S2), while the CA2 did not increase noticeably at the end of the process (proteins moving from 60 to 90mg/kg). The lower PN/PS ratio in CA1 (1.98 for CA1 vs. 2.44 for RS) or smaller increases in biochemical components of CA2 suggests that the biomass metabolism has not equilibrated, even under the same conditions as in river sand, and

moreover has not reached stabilization by the end of the process, or else colonization seems to take more time in crushed aggregates than in river sand. This difference in the biomass dynamic in the presence of different media appears to be dictated by aggregate size, i.e. average diameter(0.82mm for RS, 1.36mm for CA1, 1.6 mm for CA2) and porosity(approx. 30% for RS and 40% for CA1 and CA2). Larger aggregate grains offer less specific surface area for bacterial adhesion, nutrient availability and biomass colonization [48, 49]. The accumulation of suspended solids and biomass helps reduce pore space and provides adsorption sites for upcoming colonization. The study by Wanko *et al.* also showed an early increase (at 30 days) of biomass growth in river sand and a slower growth in crushed aggregate [26]. Another significant difference between the two supports was the variation in O2 gas within the systems (Table 1). Results indicate that crushed aggregate was still in an unsaturated condition at the top layer, even at the time of batch inlet. This finding could be explained by the greater size heterogeneity and porosity from CA1 and CA2. As such, the top river sand filter layer accounted for less O_2 gas as the feed water flowed. If this process were being continuously fed, the oxygen content could keep dropping; however, in this study, the filters were discontinuously fed and oxygen could be replenished at rest, hence no clogging was observed during the process [38]. The temporary saturation conditions in RS1 lead to a more uniform distribution of nutrients and bacterial cells in the river sand, which resulted in their rapid growth and early balance.

As observed in biochemical composition analyses, the biomass in crushed aggregate shows a difference in terms of both quantity and stabilization time. Similarly, the protein-like compounds in crushed aggregates (CA1 and CA2) were also studied by the HPSEC, and four major fractions were also recorded. The major group fractions were found to be comparable to RS1. The percentage comparisons of the four fractions between river sand and crushed aggregate at 60 and 210 days are presented respectively in Figures 10 and 11.

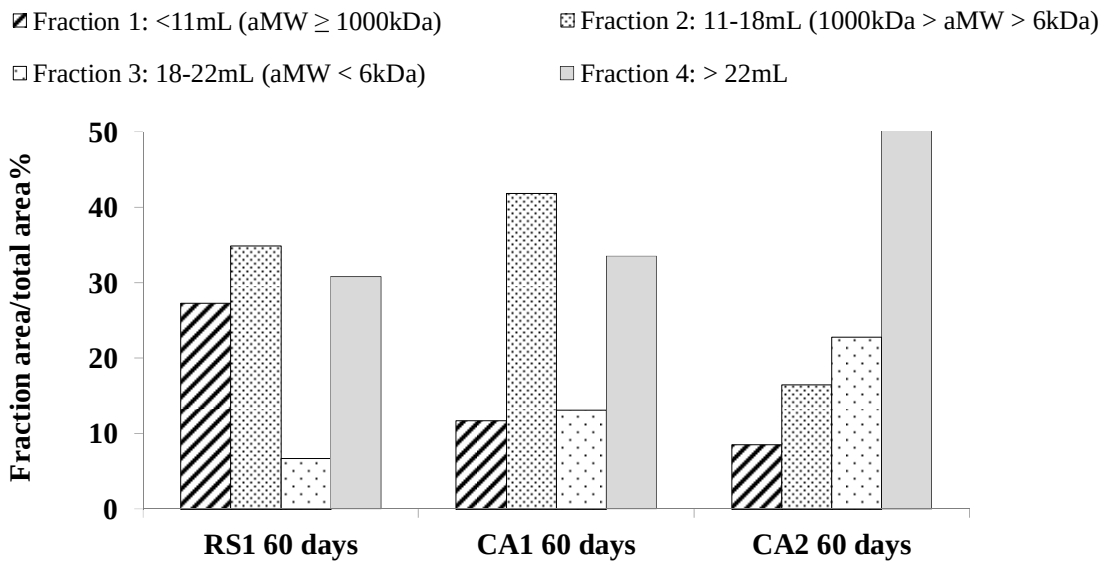

Fig.10. Comparison of major group fractions among various materials at 60 days

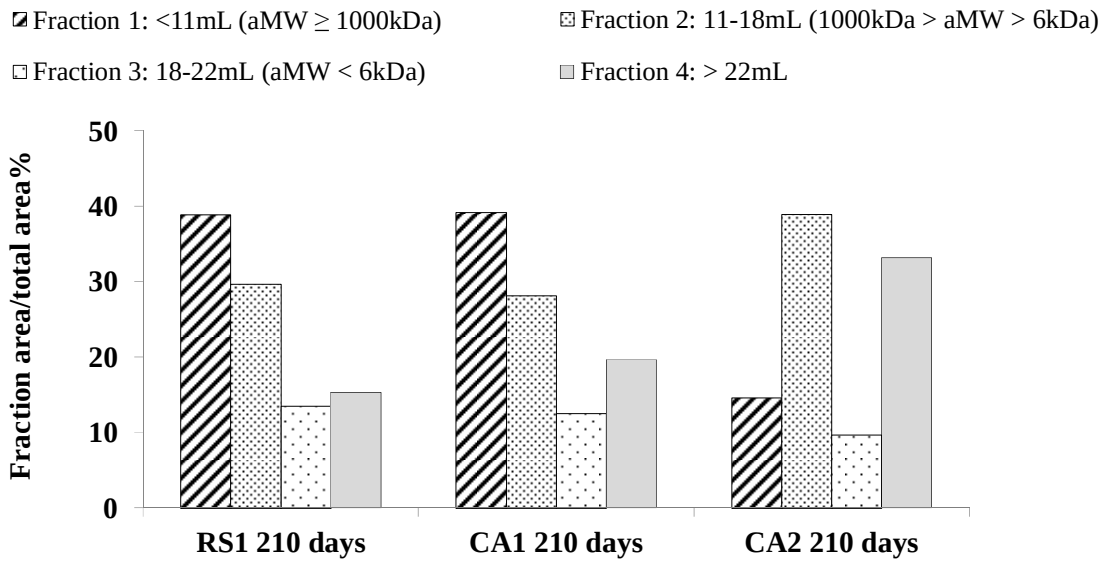

Fig.11. Comparison of major group fractions among various materials at 210 days

For RS and the two CA, the trend over time is identical: an increase in Fraction 1 and a decrease in the percentage of Fractions 3 and 4. However, the fraction partition revealed a completely different pattern for CA2. The protein-like organic matter extracted at 60 days from CA2 showed various peaks after the permeation volume (22mL), as noticed in Fraction 4 in Figure 10 (>50%), with very low proportions of Fractions 1 and 2. Both crushed aggregates presented a much lower Fraction 1 (>1000kDa) than the river sand, thus indicating that biomass growth was relatively weaker in CA1 and maybe even retarded in CA2. The relatively greater difference between Fractions 2 and 1 (42% vs. 12%) in CA1 than in RS1

(35% vs. 27%) could be due to the lower biomass protein production, which enhanced the protein-like molecules from septic effluent, but this could also be due to the protein-like synthesis during biomass growth.

CA2 at 210 days showed similar proportions to CA1 at 60 days, suggesting a more heavily delayed biomass development in this material, which may be due to its coarseness compared to that of the other materials. The similar proportions between RS1 and CA1 were observed at around 210 days of colonization, suggesting a similar evolution as in RS1, i.e. a period of stabilization for these quantities, though the biomass indicator (protein-like compounds) may endure a similar process during biomass development. HPSEC may be a suitable method for tracking the evolution/stabilization of the protein-like biomass in such processes. This method seems to be more sensitive than the percentage of biochemical compounds.

4. Conclusion

This study has been based on comparative experiments conducted between traditional filter media during 360 days: river sand and two crushed aggregates stemming from quarry output. The characteristics, especially grain size distributions, showed marked differences among these materials. CA1 exhibited considerable size heterogeneity and fine particle content. Even though great differences were observed in these materials, the purification behavior study suggested that both river sand and CA1 were effective and stable enough to remove the main pollutants of septic effluent (>95% of TSS, >90% of COD) as of Day 60.

The organic accumulations in various materials displayed their highest contents on the top layer (0~5cm), where the nutrients and bacterial cells were most abundant and where the accumulations included organic substances originating from either feed water or biomass. The subsequent biochemical study indicated an evolution in the biochemical composition of extracted organic matter, notably the increases in protein-like compounds and in PN/PS and PN/HS ratios. The HPSEC protein-like fingerprints revealed an orientation towards higher MW compositions after 210 days of colonization for the RS and two CA extracted biomass.

With the crushed aggregates tested herein (namely CA1 and CA2), the main difference observed was the decrease in quantities of biochemical components and the longer time required to establish biomass stability, especially for CA2. The biomass stability monitoring tool consists of: the PN/PS and PN/HS ratios and the percentages of >1000kDa fraction with

SEC fingerprints. This difference should be exposed by the various material characteristics: increase in porosity and grain size, along with size heterogeneity during the process resulting in a longer biomass stabilization period.

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3.3. (Article 3): Dynamics of extracellular polymeric substances (EPS) derived from the biofilm in on-site wastewater filtration reactors

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Abstract

Extracellular polymeric substances (EPS) from biofilters (packed with river sand (RS) and crushed aggregate (CA)) installed in on-site wastewater treatment systems are characterized over 360 days of an enrichment process and within the bed thickness. Biochemical component contents are monitored; moreover, humic and protein-like compounds are characterized by means of Size Exclusion Chromatography (SEC) coupled with fluorescence. During the biomass enrichment phase, EPS biochemical components increase at the top of the biofilter (protein enrichment factor >70%). The protein-like components exhibit a very high MW fraction (apparent molecular weight (aMW) >1,000kDa), which may contribute to cell aggregation. Humic-like substances show similar SEC fingerprints to those of the feed water (aMW<6kDa) and are perhaps being metabolized at around Day 210 (as evidenced by a lower aMW). Only the dynamic polysaccharide partition in EPS differs between biofilters, with an increase for CA and a decrease for RS. Within the filtration bed thickness, lower biomass with a higher EPS content is observed, and the polysaccharide fraction increases by a factor of 2. Protein-like components exhibit a very high MW fraction of a lower magnitude.

Keywords: packed bed filtration, biofilm, EPS, proteins, humic-like substances, SEC coupled with fluorescence.

(Sera soumis à Bioresource Technology)

1. Introduction

On-site wastewater treatment systems (OWTS) are typically located in rural areas without connections to a municipal wastewater treatment network. The most common configuration involves a septic tank offering pretreatment by anaerobic digestion, whereby the pretreated (septic) effluent is spread over soil infiltration fields, or more commonly, filtration beds packed with river sands or similar materials. Biomass exits the filtration reactors in the form of a biofilm, which protects the microbial cells that are irreversibly attached to the substratum by embedment in a matrix of extracellular polymeric substances (EPS) (Donlan and Costerton, 2002). The biomass or biofilm population serves an important role as purification agent of organic nitrogenous pollutants. The structural characteristics of biofilm (i.e. the EPS matrix) however also take into account the environment of a porous medium and tend to reduce the limited space between pores until biofilm development reaches stabilization. Yet the presence of biofilm is also considered to "clog" porous media, as provided by river sand or other materials in filtration reactors, e.g. polysaccharides (Zhao et *al.*, 2009; Kim *et al.*, 2010).

The extracellular matrix of biofilm contains a "gel"-like structure and is composed of major biochemical components either tightly or loosely bound to the cells, such as proteins, polysaccharides, nucleic acids, humic-like substances (Frølund *et al.*, 1996; Flemming and Wingender, 2001a), metabolic wastes, and absorbed substrates and minerals (Wingender *et al.*, 1999; Tsai et *al*., 2008; D'Abzac *et al*., 2011b). The roles of EPS are believed to include, without being limited to, the following: structural formation and maintenance of aggregates or biofilm; increase in substrate diffusivity; and aggregate morphology in correlation with EPS hydrophobicity. It can still be argued whether EPS production reflects higher or lower microbial activity. The excretion of intracellular material might be a survival mechanism under unfavorable conditions, such as enhanced extracellular enzymatic activity and/or other mechanisms facilitating cell aggregation (Liu *et al.*,2004).

EPS proteins constitute a group of molecules that perform critical functions in the biofilm (Arnosti and Jørgensen, 2003; Frølund *et al*., 1996; Pell and Nyberg, 1989). Proteins also possess a high molecular weight (45~670kDa) and may be associated with other macromolecules, such as polysaccharides (Görner *et al.*, 2003; Bourven *et al*., 2014). The molecular weight partition of the EPS protein changes depending on the state of the biofilm (Martinez *et al*., 2004; Zhang *et al.*, 2007). The polysaccharides in an EPS matrix exist in the form of either a capsule covalently associated with a cellular membrane (lipopolysaccharides) or slime weakly coupled with the cells (Kumar *et al*., 2007). Polysaccharides are hydrophilic

molecules that tend to retain water and assist the microbial cells in retaining nutrients (Wingender *et al*., 1999; Imai *et al*., 1997). The humic-like substances (HS-like) are believed to be exogenous compounds captured in the biofilm before undergoing repolymerization (Francioso *et al*., 2002). However, Guo *et al*. (2011) found that humic acid-like substances detected by means of three-dimensional spectrofluorimetry appear during aerobic granulation. Some authors have pointed out that another pathway exists for the formation of these HS-like: following the degradation of macromolecules (like carbohydrates and proteins) under microbial attack, the refractory compounds or biopolymers are selectively transformed to produce the high MW precursor of humin, subsequent to which the molecules become smaller during the additional oxidation process (Vanloon and Duffy, 2005). Nucleic acids used to be considered as the indicator of cell lysis during extraction; later research however has found that many microorganisms secrete extracellular nucleic acids (Steinberger and Holden, 2005).

Protein and polysaccharide EPS contents had been tracked as a potential indicator of biofilm state: the protein content increases with the formation and stability of aerobic granules (Zhang *et al.*, 2007), whereas Ahimou *et al*. (2007) showed that the level of biofilm cohesive energy is strongly correlated with polysaccharide content.

Studies on EPS from bioprocess systems have been extensively conducted in an attempt to establish a connection between reactor performance and EPS physicochemical features. This performance includes sludge settling and flocculation capability (Liu and Fang, 2003), biofilm and granular formation (Liu et *al.*, 2004), and membrane fouling (Her *et al*., 2007). Accordingly, the EPS study on OWTS is focused on two topics: biofilm formation, and clogging prevention. However, research carried out on biofilm extracted from filtration reactors has mainly dealt with biological clogging tied to biomass expansion and the microbial community on the filter medium, i.e. total organic accumulation(Campos *et al*., 2002; Zhao *et al.*, 2009), in including the main biofilm components. Proteins and polysaccharides exhibit a linear correlation with operating time and a decreasing abundance with depth (Regusa *et al*., 2004). Among these organics, the loose "slime"-like exopolymers appear to cause the drop in hydraulic conductivity while the cells exert no effect on clogging (Vandevivere and Baveye, 1992; Ronner and Wong, 1998). None of these studies have ever covered the evolution of EPS composition and characteristics. The aim of this work therefore is to provide in-depth information regarding EPS characteristics in relation to the various stages of the biofilter enrichment process as well as to bed thickness. For this study, two types of packing materials, river sand and crushed aggregate, have been used.

2. Materials and methods

2.1. Batch experiments (cylindrical filtration reactors)

Two filtration reactors, 70 cm high and 30 cm in diameter with sampling ports, were packed with river sand and crushed aggregate, respectively (see Fig. 1). These reactors were then fed with septic effluent that had been stored in a mixed tank with replenishment every 7 days. The reactors were fed discontinuously, at a hydraulic loading rate of 12 cm/day in 10 daily batches. The feed-rest condition induces liquid passage, which may involve varying the O_2 level. The O_2 gas variations in each reactor were estimated by means of an optical oxygen sensor (OXROB10, Fire Sting O2, with Pyroscience sensor technology). A saturated/unsaturated gas phase alternation was found with the river sand reactor, but the reactor with crushed aggregate showed less variation in oxygen gas.

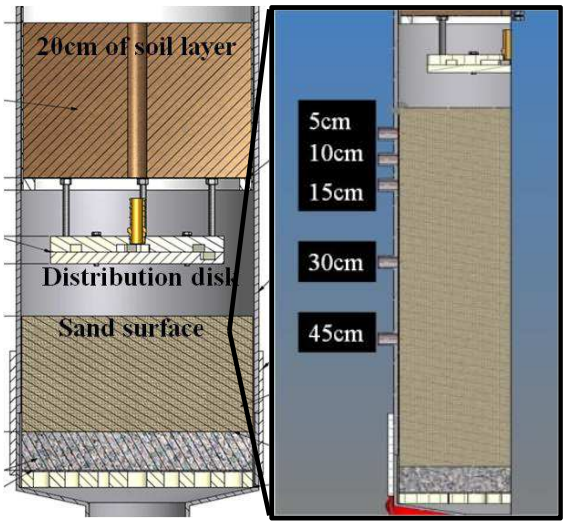

Figure 1 : Filtration reactor with sampling ports

2.2. Packing material characteristics

The filter materials were analyzed before column packing, given that the river sand and crushed aggregate differed in composition and involved distinct treatment processes at the quarries. The two materials were studied and compared in terms of particle distribution, and both mineralogical and physical characteristics. Their hydraulic and hydrodynamic properties were assessed using two post-packing filtration reactors (Liénard *et al.*, 2001). These characteristics are listed in Table 1.

2.3. Feed water: Septic effluent characteristics

The feed water was collected from the septic effluent settling tank, with all main characteristics being monitored throughout the operating period. The average and extreme values of each characteristic are reported in Table 2.

2.4. Total biomass and EPS extraction

Three samplings and extractions were carried out on Days 60, 210 and 360 for the top layer of the medium. At the end of the operating period (i.e. 360 days), extractions were also performed on the various layers in the two filtration reactors (5 cm, 10-15 cm and 30 cm). The total biomass was extracted as quickly as possible (< 24 h) both by heating to 80°C during 30 min for the colorimetric analysis (proteins, HS-like, polysaccharides and nucleic acids) and by sonication at 4°C during 60 min for the Size Exclusion Chromatography (SEC) fingerprint analysis (Zhang *et al*., 2009; Bhatia *et al*., 2013). The EPS was extracted by sonication at 4°C over 5 min for both the colorimetric analysis and SEC fingerprint analysis. The nucleic acids were assessed with a series of ultrasound extractions of increasing duration: after 5 min, the nucleic acid contents exhibited a significant increase within the filter media samples.

Table 1: Characteristics of river sand (RS) and crushed aggregate (CA) as the main packing materials

Material	River sand (ref. RS)	Crushed aggregate (CA)
Particle size distribution characteristics		
Effective size D_{10} (mm)	0.38	0.17
Average diameter D_m (mm)	0.82	1.36
Uniformity coefficient (D_{60}/D_{10})	2.8	10.0
Fine particles % (< 0.08 mm %)	0.4%	5.0%
Physical and chemical characteristics (average values; n=5)		
Real density (kg/m^3)	2525 (±42)	2438 (±111)
Porosity (%) [min; max]	[30%; 33%] (±1.5%)	[38%; 41%] (±1.7%)
Specific surface area (m^2/kg)	4.04	2.78
Hydraulic characteristics (average values; n=5)		
Estimated hydraulic conductivity (m/s)[min, max]	[8.25~9.53] $\times 10^{-4}$	[2.79~2.88]$\times 10^{-4}$
Water retention capacity after drainage (static water %)	8.4%	12.7%
Hydraulic Residence Time (hours) (n=1)	35	93
Variation of O_2 gas level at 10 cm [min, max%]	[11, 20]%	[19.2, 19.8]%

Mineralogical composition (mg/kg of material)		
Ca	< 5	795
Mg	74	1535
Na	< 5	295
K	1370	2356

Table 2: Feed water characteristics

Parameter	Average value	[min; max]
pH (5 tests)	7.1	[6.6; 7.5]
TSS (mg/L, 18 tests)	39 (±11)	[20; 66]
VSS (mg/L, 1 test)	23	-
COD (mgO/L, 18 tests)	372 (±100)	[231; 572]

2.5. Total biomass, feed water and EPS characterization

The biochemical components of biomass, EPS and feed water were quantified by employing colorimetric methods: proteins and humic-like (HS-like) substances using the modified Lowry method in introducing Folin's reagent (Frølund *et al*., 1996); polysaccharides using Dubois' method; and nucleic acids using Burton's method (Burton, 1956; Dubois *et al*., 1956).Nucleic acids are present for the purpose of EPS extraction control (Liu *et al.*, 2003). Considering the relatively weak content of nucleic acids in the extracted EPS, it appears that the EPS extracted during this study was not contaminated by significant amounts of intracellular materials (Comte *et al.*, 2006).

The apparent molecular weight (aMW) distributions of biomass proteins and humic substances were analyzed by means of High Pressure Size Exclusion Chromatography (HPSEC) (Merck Hitachi LA Chrom Chromatograph), coupled with fluorescence detection. The high molecular weight separation was performed with the Agilent column (BioSec, 300A, 5-1,250 kDa), while the low MW separation made use of the BioSec 100A Agilent column (0.1-100 kDa). All columns were placed in series for the separation improvement step (Bourven *et al*., 2014). The mobile phase was composed of a 150-mM NaCl and 50-mM phosphate buffer at pH 7.0. The MW were calibrated using six proteins or amino acids, with MW values of: 440000, 155000, 69323, 5777, 670, 362 and 181 Da (matching respectively ferritine (Sigma), immunoglobulin G from human serum (Sigma), albumin from bovine serum (Sigma), insulin from porcine pancreas (Fluka), thyroglobulin, thyrotropin-releasing

hormone (Fluka), and tyrosine (Fluka)). For the mass calibration curves, the logarithm of molecular mass (Log (MW)) has been plotted as a function of the elution volume (mL), i.e.:

$$\text{Log (MW)} = -0.3164\ Ve + 9.4676\ (R^2 = 0.982)$$

with MW being the molecular weight (Da), and Ve the elution volume (mL). The permeation volume determined with NaN_3 equaled 22 mL. The Excitation/Emission (Ex/Em) wavelength fluorescence detection for protein-like (protein tryptophan-like) substances was found to be 222/330 nm, while for humic-like substances (HS-like) the value amounted to 350/460 nm. These wavelengths were derived with the Shimadzu RF-5301 PC spectrofluorometer. The two coupled Ex/Em wavelengths corresponded to the maximal fluorescence in the protein tryptophan-like and HS-like, as defined by Chen *et al*. (2003).

The chromatograms were established from both the fluorescent intensity (volts) and elution volume (mL). Several fractions were identified as the elution volume interval (e.g. 12~18 mL). The fractional area percentage calculation was based on the ratio of the chromatogram area of one fraction to the total chromatogram area. This calculation was performed using the Origin 6.0 software package. The fractional (F) area percentage is defined as follows:

F% = 100 × Area of fraction / Total area of chromatogram

3. Results and discussion

3.1. Comparison of biochemical characteristics of organic matters of effluent and of EPS extracted from filter material

The feed water (effluent) was responsible for importing a variety of organic matter, some of which was caught by the filtration medium. This organic matter will subsequently be used as substrate; however, its presence is inevitable and influences biofilm component characterization. The EPS were extracted and the fractions of each major biochemical component were compared to those of the feed water (Fig. 2). For this comparison with feed water, EPS extracted from RS has been used.

Quantitative results indicate that biomass and EPS display a distribution of biochemical components different from that of feed water: protein proportions in the biomass or EPS show a rise (20% in feed water vs. approx. 40% and 50% respectively in EPS and total biomass). The EPS from river sand is composed of a similar ratio between proteins and HS-like

compounds at both 210 days (7 mg proteins vs. 6 mg HS-like/kg material) and 360 days (30 mg protein vs. 38 mg HS-like/kg material). Both the nucleic acids and polysaccharides are less significant components in the EPS than in feed water (Fig. 2a). The proportions of biochemical components (Fig. 2b) also exhibit distinct distributions between EPS and total biomass, with higher proteins and lower HS-like proportions for biomass extraction, since the total biomass includes intracellular as well as extracellular proteins. In EPS on the other hand, the proportion of HS-like exceeds proteins at 360 days. This increase in EPS proteins may be due to bacterial production or protein-like molecules; also, it might stem from the environment or from cell lysis (Wingender *et al*., 1999; Flemming and Leis, 2001).

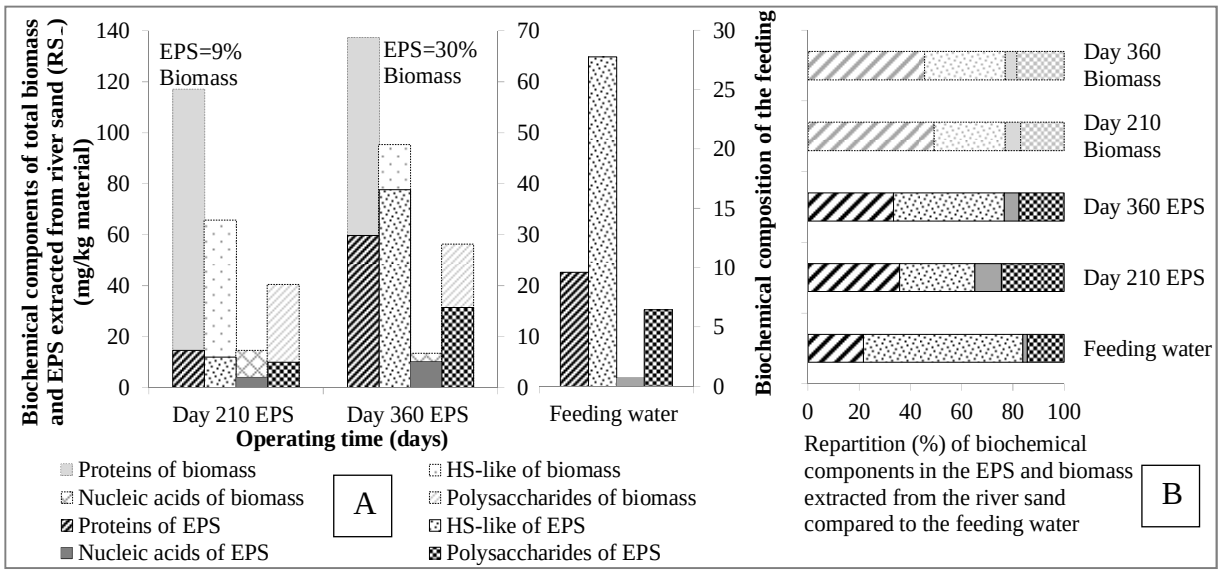

Figure 2 : Biochemical component contents (A) and repartition (B) of EPS, biomass from the top layer of the biofilter (RS) at both 210 and 360 days of colonization and feed water

Other analytical tools are needed to compare the qualitative characteristics of EPS components. As the main extracellular matrix components, the PN-like and HS-like compounds were examined using SEC fingerprints coupled with fluorescence detection. The resulting chromatograms are shown in Figure 3.

In Figure 3a, the HPSEC protein-like fingerprints present similar MW distributions between the EPS and biomass of the river sand sample: the aMW of protein-like substances (PN-like) spikes from < 6 kDa to > 1000 kDa, although this distribution differs in comparison to feed water. Several fractions can be described, namely:

- Fraction of very high MW (VHMW) (10mL<Ve<12mL): one prior peak with an apparent MW (aMW) ≥1000kDa;
- Fraction of high MW (HMW) (12mL<Ve<14mL): groups of peaks not well distinguished from one another and often with two peaks shown (1000kDa>aMW> 109kDa);
- Fraction of low MW (LMW) (14mL<Ve<16mL): groups of peaks distinct from one another (109kDa>aMW> 25kDa);
- Fraction of very low MW (VLMW) (18mL<Ve<22mL): one or several peaks depending on the sample (aMW<6kDa). For an elution volume >22mL, the molecules are eluted beyond the total permeation volume of the column.

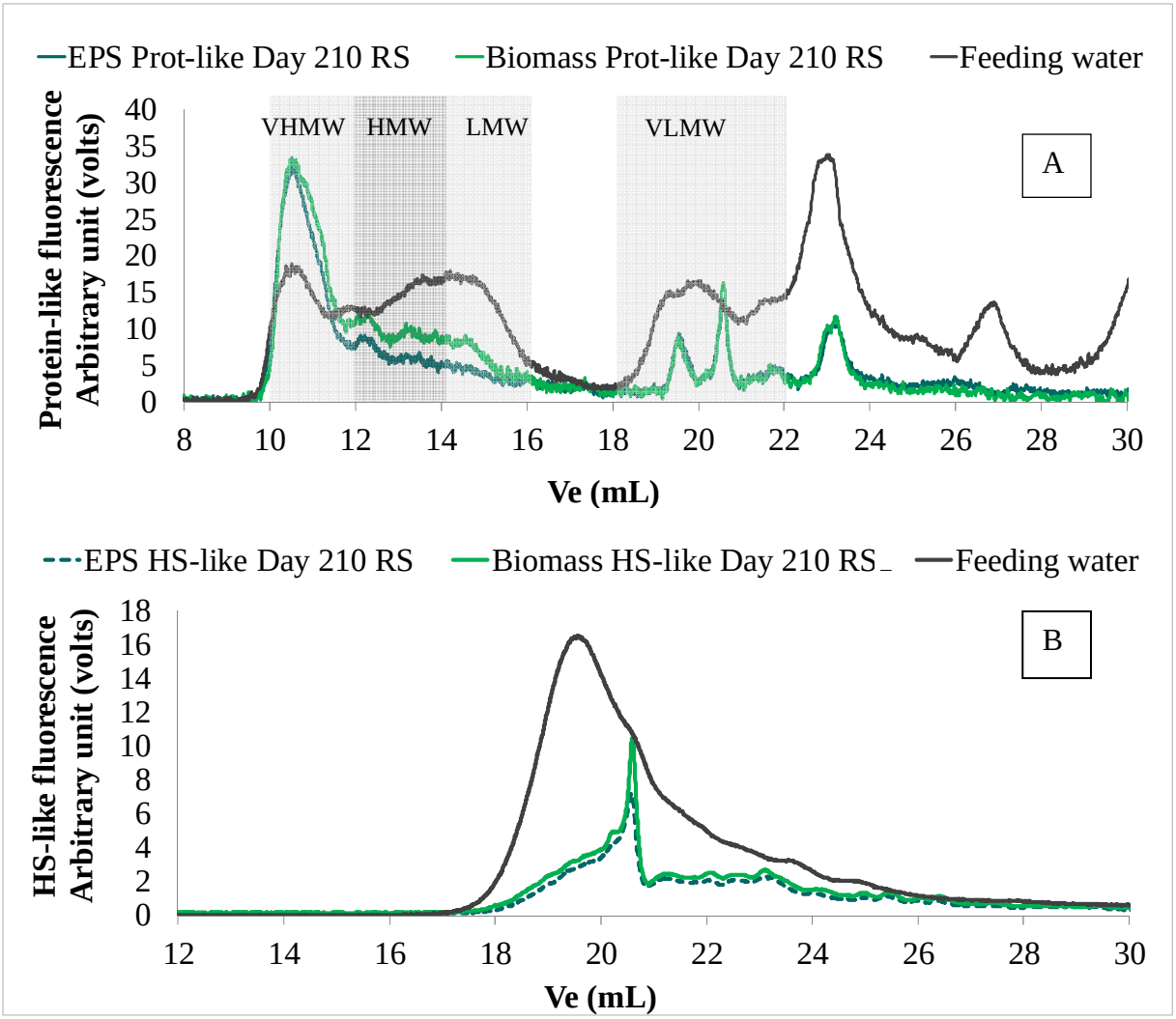

Figure 3: Protein-like (PN-like) (A) and Humic-like (HS-like) substances (B) HPSEC fingerprints of EPS and biomass extracted from biofilter (RS) at 210 days compared to the feed water

The percentages of the fingerprint fractional area have been calculated and displayed in Figure 4.

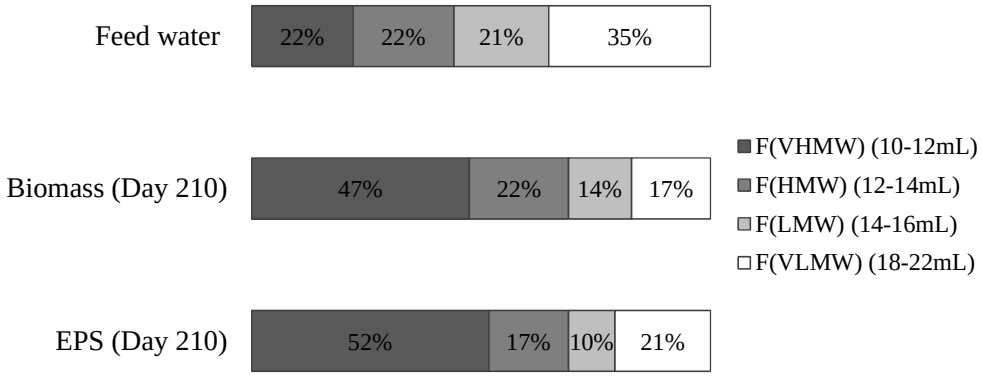

Figure 4: Protein-like SEC fingerprint fraction area percentage (%) of biomass and EPS from the river sand (RS) on Day 210, compared to the feed water percentage

These characterizations reveal a similar distribution between biomass and EPS: a higher VHMW fraction with aMW > 1000 kDa, while the feed water contains mainly very low MW PN-like eluted after the permeation volume (VLMW fraction). The biomass and EPS exhibit similar proportions of VHMW fraction, which could result from the polymerization of PN-like compounds due to their association with other types of molecules within the extracellular biofilm matrix (Bourven *et al*., 2014). The biomass PN-like fingerprint reveals higher HMW and LMW fractions when compared with those of EPS: these fractions may be PN-like molecules located in cells (intracellular protein-like).

In contrast, compared to proteins, the HS-like are primarily molecules with a weak MW (Ve > 18 mL: aMW < 6 kDa) (Fig. 3b). Bhatia *et al.* found that the HS-like in EPS of an aerobic granule was less than 6 kDa in the MW fraction (Bhatia *et al*., 2013). The HS-like also present similar fingerprints between EPS and biomass extracted from river sand. Since humic compounds are not produced by biofilm cells but rather captured from the environment, the presence of HS-like compounds should be extracellular. The similarity between EPS and biomass HS-like fingerprints, along with the differences in PN-like fingerprints of EPS and biomass, has confirmed that the biomass extraction includes both intracellular and extracellular components. The shape and peak maxima of the HS-like fingerprints of EPS and biomass on Day 210 however differ from those of feed water (feed water: $Ve_{maximum\ peak}$ = 20 mL; EPS and biomass: $Ve_{maximum\ peak}$ = 21 mL). The hypothesis may thus be forwarded that the river sand biofilm selectively adsorbed humic-like compounds

from the feed water at a different developmental state, and/or the HS-like modification (metabolism) took place in the biofilm due to degradation (catabolism) (Volks *et al*., 1999; Vanloon and Duffy, 2005).

The above findings show that the biochemical components of total biomass and EPS differ from those of feed water, with a higher PN content, in particular very high MW PN-like compound (> 1000 kDa) percentages. Furthermore, the total biomass contains two slightly higher MW fraction percentages (HMW fraction: 1000 kDa ~ 109 kDa and LMW fraction: 109 kDa ~ 25 kDa) than the corresponding EPS percentages. These fractions could correspond to intracellular proteins.

The evolution in EPS has resulted from the filtration reactors at two scales, i.e.: evolution during process implementation, and evolution as a function of the vertical profile (depth) of the reactors. Two materials from validated biofilter treatment processes (river sand (RS) and crushed aggregate (CA)) can yield observations of biofilm evolution, due to the fact that EPS is essential to biofilm survival.

3.2. Evolution of the biochemical characteristics of EPS vs. operating time

1. Biochemical composition:

The evolution of EPS percentages in the biomass extracted from the top layer of each filtration reactor on Days 60, 210 and 360 (as calculated from the data available in Fig. 5) is presented in Table 4. During the implementation process, total biomass increased in the two materials, by factors of 3 and 2 for RS and CA respectively. This biomass increase corresponds to the protein content evolution (95% and 71% factors for RS and CA, respectively), which serves as the biomass activity indicator (Di Iaconi *et al.*, 2006) (Fig. 4). Biomass implementation seems to reach stabilization for RS with a growth factor of 1.3 and 1.8 respectively for biomass and especially for protein. This same trend is observed in the two materials with an increase of EPS percentage in the biomass, from approx. 16% to 30% in RS and from 21% to 32% in CA. It is still open to debate whether EPS production reflects higher or lower microbial activity, though it is usually reported that during the exponential growth phase, EPS content increases with time yet decreases once the stationary phase has been reached (Jia *et al.*,1996). Both EPS percentages exhibit a slight decrease between Days 60 and 210 (Table 4).This decrease is simply due to the drop in HS-like substances (Fig. 4) from 7.1 to 5.9mg/kg material in RS and from 15.1 to 7.1mg/kg material in CA. As explained in the

previous section, humic substances, which are generated from effluent, do not get synthesized but might be metabolized by biofilm cells around Day 210 (Volks *et al*., 1999).

The EPS percentage in the biomass however remains somewhat higher for CA, especially at the beginning of the implementation step; at this same time, a higher biomass increase for RS than for CA is revealed. The biomass content evolution could be due to a higher specific surface area for RS than for CA (Table 1) or to a higher speed of development. As observed from the material characteristics (Table 1), the CA reactor displays an extended hydraulic residence time and water retention capacity, both of which may due to the fine particle content. The static liquid phase may lead to microenvironments with a weak renewal of nutrients and/or accumulated residues. The hydraulic and hydrodynamic variations could result in low biomass activity (i.e. total protein content in CA) but a high proportion of EPS in the reduced biomass (as is the case with CA). This low substrate content could favor EPS synthesis (Gao *et al.*, 2008; Nichols *et al.*, 2004). Starvation would be the preferred hypothesis over hydrodynamic influence for the increase in EPS content from aerobic granules growing on a zeolite material biofilter (Di Iaconi *et al.*, 2006). Moreover, changes in environmental conditions could induce a shift in microbial community and, subsequently, more EPS-producing content (Gao *et al.*, 2008; Liu *et al.*, 2004). Throughout all days of the process, total -N removal (%) is greater (by a factor of 1.5) for RS than for CA, which means that denitrifying bacteria are less active or less present in the CA biofilter.

Table 4: Biomass evolution and EPS percentage in the biomass extracted from the top layer of each filtration reactor on Days 60, 210 and 360

Material	River sand (RS) - top layer			Crushed aggregate (CA) - top layer		
Time	60 days	210 days	360 days	60 days	210 days	360 days
Biomass (mg/kg)	96	238	303	123	141	251
EPS%	16	9	30	21	17	32

The biochemical contents of EPS and biomass extracted from the top layer of the two reactors on Days 60, 210 and 360 are presented in Figures 5 and 6:

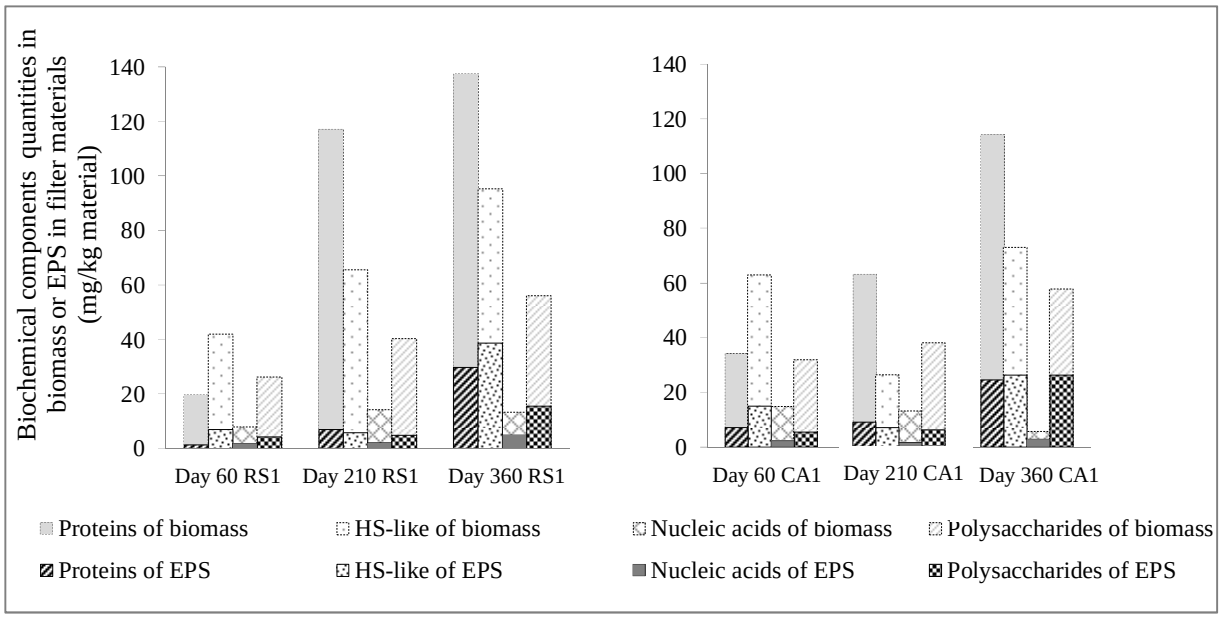

Figure 5: Evolution in biochemical component quantities of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA)

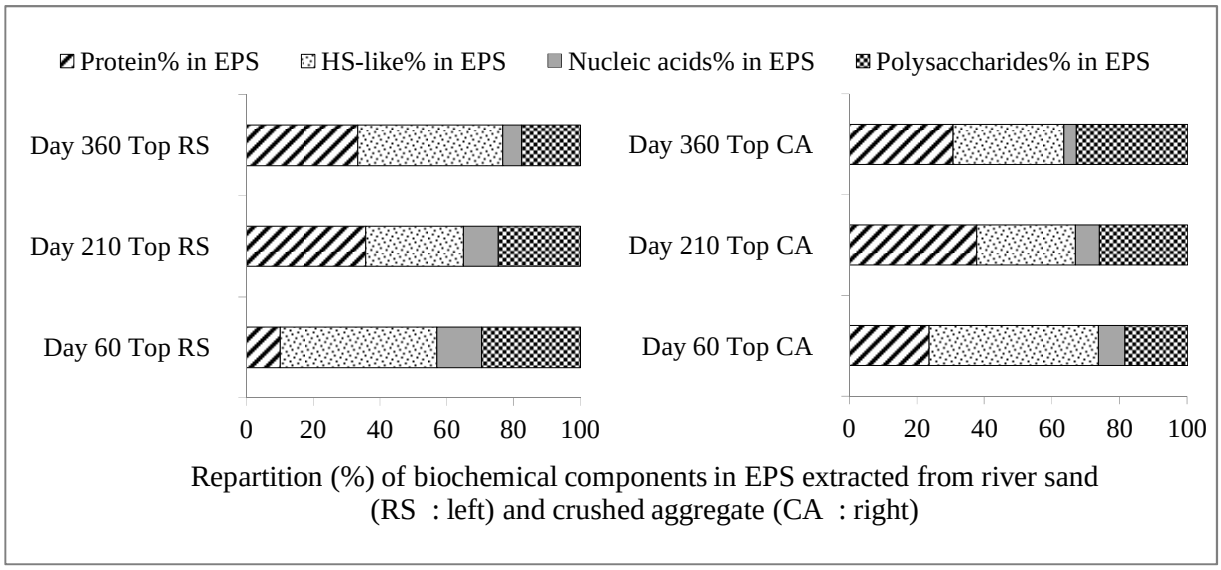

Figure 6: Evolution in biochemical component repartition of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA)

The evolution in biochemical component quantities of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA) (Fig. 5) suggests that the evolution in extracellular biochemical components undergoes the same process for both materials. Enrichments have been indicated in: PN-like (with growth factors of 19 and 3 for RS and CA respectively), HS-like (by factors of 5 and 2 for RS and CA respectively), and PS (factors of 3 and 5 respectively) (Fig. 5). The majority of organic matter in an extracellular matrix is

composed of proteins and humic compounds (Fig. 6), which are found in greater proportions in active sludge (Frølund *et al.*, 1996). Zhang *et al*. (2007) observed that the stability of aerobic granules during biofilm granulation is mediated by PN, while for Ahimou *et al.* (2007) cohesiveness is correlated with PS.

When comparing the evolution of proportions in biochemical components (Fig. 5), this same trend is identified with increasing PN percentages (RS: 10~33%; CA: 24~31%) and decreasing HS-like (RS: 47~43%; CA: 50~33%). Zhang *et al*. (2007) described such enrichment in PN content of EPS during aerobic granule formation and proposed that an increase in PN might enhance neighboring microbial cells and form a cross-linked network by attracting organic and inorganic material (Liu *et al*., 2004). The percentage of PS however becomes inverted, with an increase (18~33%) and decrease (30~18%) for CA and RS, respectively. Hence, EPS in biofilm does not necessarily develop in the same way on the two filter materials. The difference in EPS biochemical composition may be explained by the differing conditions generated in the materials, i.e. porosity (both external and internal), fine particle contents and reactor hydrodynamics. Moreover, the mineralogical composition (Table 1) differs substantially with crushed aggregate: higher calcium content leads to a greater presence of divalent cations at the material surface, with the possibility that divalent cations bind with extracellular PS-alginate-like via an ionic link, thus resulting in a complex "egg-box" configuration (Sobeck and Higgins, 2002; Lin *et al*., 2010). Stabilized PS are less influenced by effluent and/or less metabolized.

In light of the above discussion, similar trends can be observed for PN- and HS-like, as recorded in both materials. A follow-up qualitative comparison of PN-like and HS-like substances was conducted by examining the MW distribution.

2. Protein and HS-like HPSEC fingerprints:

In examining the major biofilm components, a further qualitative study on the MW distribution by HPSEC fingerprints of protein-like substances between filter materials will be described and the chromatogram displayed in supplementary data. Moreover, Figure 7will present the fractional area percentages.

As observed in the PN-like fingerprints (S1, supplementary data), their peak number and elution volume differ with incubation time. This finding indicates that diverse molecular

structures have occurred as a result of biomass enrichment. Similarly, in Figure 7, the increase in the VHMW fraction and decrease in the VLMW fraction of PN-like fingerprints were noted in both filter materials after 60 days of operations. This MW distribution shift may be due to the production of new PN-like and their polymerization with other organic molecules during the enrichment step. Since extracellular proteins contain a considerable amount of enzyme, it is assumed that the extracellular enzymatic activity might change with the increased biofilm implementation activity. This increase in the VHMW fraction may be correlated with the aggregation of bacteria in biofilm during biomass enrichment.

The RS showed a relatively stable evolution in fractional area percentages from Day 60 to Day 210. A decrease in the VHMW fractional proportion coupled with an increase in the LMW fractional proportion could be noticed on Day 360. The decrease in the VHMW fractional proportions may be explained by a degradation in existing compounds at the mature biofilm state (Nielson *et al*., 1996). For CA on the other hand, the VHMW fraction is increasing during all 360 days of biomass enrichment, thus suggesting a late increase in molecular polymerization. This late evolution in the MW shift distribution of PN-like molecules in CA may be caused by somewhat delayed biofilm development in such material; this pattern was also observed in the biochemical component evolution for total biomass (Fig. 4). Let's notice that between Days 210 and 360, in both materials, the HMW fractional distributions became smaller, which may be due to the degradation of PN-like for cell catabolism.

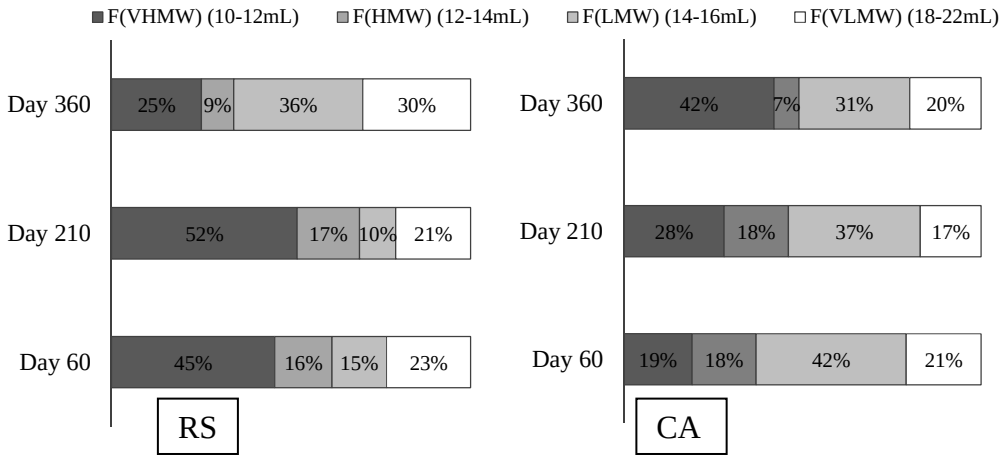

Figure 7: Percentage of areas for PN-like fingerprint fractions of the EPS extracted from RS and CA during the operating period

The HS-like fingerprints of the EPS extracted from the two materials have also been described from Day 60 to Day 360. These results are presented in Figure 8.

As mentioned in the previous section, HS-like were confirmed to possess weak MW (< 6 kDa) on Day 360 in both materials. The majority (i.e. major peak) fraction varied similarly between the two filter materials over the operating period. The small peaks located between 20 and 22 mL on Day 60 had moved to between 20 and 21 mL by Day 210 and wound up being positioned at 20 mL on Day 360. Let's note that, for CA especially, the area under peak evolution exhibits the same trend as the HS-like content observed in Figure 4 over time; during this process, the aMW of HS-like increased. As mentioned in the previous section, the HS-like first absorbed in the biofilm might thus be the humic compounds of very weak MW or the HS-like modified by the biofilm; furthermore, the HS-like fingerprints of EPS in the two materials were similar to those of the feed water. During the first process interval, either: i) in considering the hypothesis of selective adsorption of HS-like from the effluent, smaller molecules are perhaps more easily and quickly adsorbed onto the biofilm; or ii) in considering the hypothesis of metabolism of HS-like by cells, HS-like from the environment are no longer used after implementation (hence no longer being degraded).

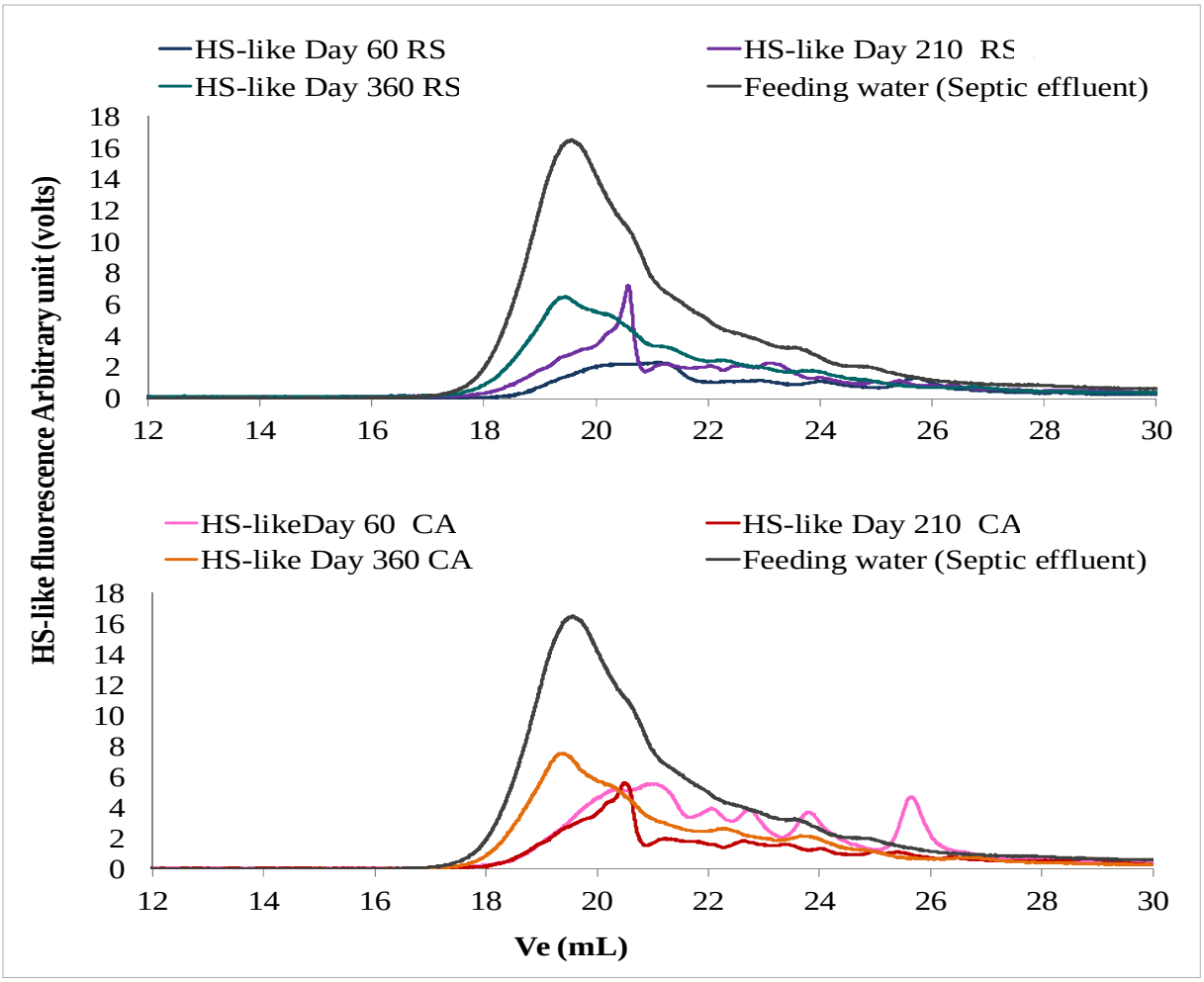

Figure 8: Evolution in HS-like fingerprints of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA)

3.3. Evolution of EPS biochemical composition with bed thickness

To observe the impact of packing materials on biofilm evolution in the deeper part of the filtration reactors, i.e. where the environment differs from that of the top layer (less rich in substrates), a follow-up study of the vertical EPS distribution in filtration beds with different filter materials was conducted after 360 days of operations using the same analytical tools.

1. Biochemical composition:

The evolution of biomass and EPS percentages in the biomass extracted from layers 0-2 cm, 5 cm, 10-15 cm and 30 cm in each biofilter on Day 360 (as calculated from the data available in Fig. 9) is shown in Table 5. The biomass in both materials decreased with filter medium depth; moreover, a higher biomass in RS from the top to the 10-15 cm layer and a

lower biomass at 30 cm compared to CA were observed. This same trend had been identified for both types of material packing: increasing EPS percentage in biomass, coupled with a declining biomass over the depth. The EPS percentage remained constant in the same layer and on the same order of magnitude for both materials: about 30% for the top, and increasing with depth (up to 38%).

Table 5: Evolution of biomass and EPS% from biomass extracted over the depth of each reactor

Material	River sand (RS) on Day 360				Crushed aggregate (CA) on Day 360			
Depth (cm)	0-2 cm	5 cm	10-15 cm	30 cm	0-2 cm	5 cm	10-15 cm	30 cm
Biomass (mg/kg)	303	169	125	45	251	166	71	63
EPS%	30	25	27	39	32	30	44	37

The biomass also decreased with depth due to fewer cells when less of the substrate had penetrated into the depth. Like for the previous study (EPS vs. time of evolution process), total biomass components appear in higher contents from the top to the 10-15 cm layer in RS than in CA. EPS components show a similar scale, except with slightly more polysaccharides in CA. The increase in EPS percentage above this level, compared to its total biomass over the filter medium depth, could be due to: i) the downward shift through the depth of the weakly bound soluble EPS; ii) less substrate and/or bacteria community shift (more nitrifying bacteria, which are autotrophic); and iii) a new C/N ratio over the deeper part of the beds. Gao *et al.* (2008) also described how nitrifiers (which appear due to the new C/N ratio) tend to produce more EPS than heterotrophs within the upper media of an aerobic biofilter. Moreover, a low C/N ratio (as is expected with depth in our study) was reported by Durmaz *et al*. (2001) to induce a low EPS content when compared with a ratio of 40. The fine particles in CA should be more noticeable over the depth than in the top layer, which may also lead to a higher EPS percentage by structurally influencing the biofilm (Vieira and Melo, 1995).

The proportions of biochemical components have changed in the deeper part of the filtration beds (Fig. 10, 30 cm). CA reveals lower quantities in proteins than in polysaccharides of EPS (in both the absolute and relative fractions), with the PN/PS ratio decreasing from 1 in the top layer to 0.1 at 30 cm. For the RS material, PN/PS ratios were typically higher, with a value of 2 in the top layer, around 1 from 5 to 15 cm and 0.5 at 30 cm.

In deeper parts, bacteria feature less substrate. Furthermore, the proportions of polysaccharides in EPS, which increase with medium depth in CA and RS, might be due to the lower accessibility of nutrients or change in bacterial community, or a new C/N ratio, or various physical environments. Durmaz *et al.* (2001) however showed that a lower C/N ratio induces an increase in PN content and a decrease in PS content.

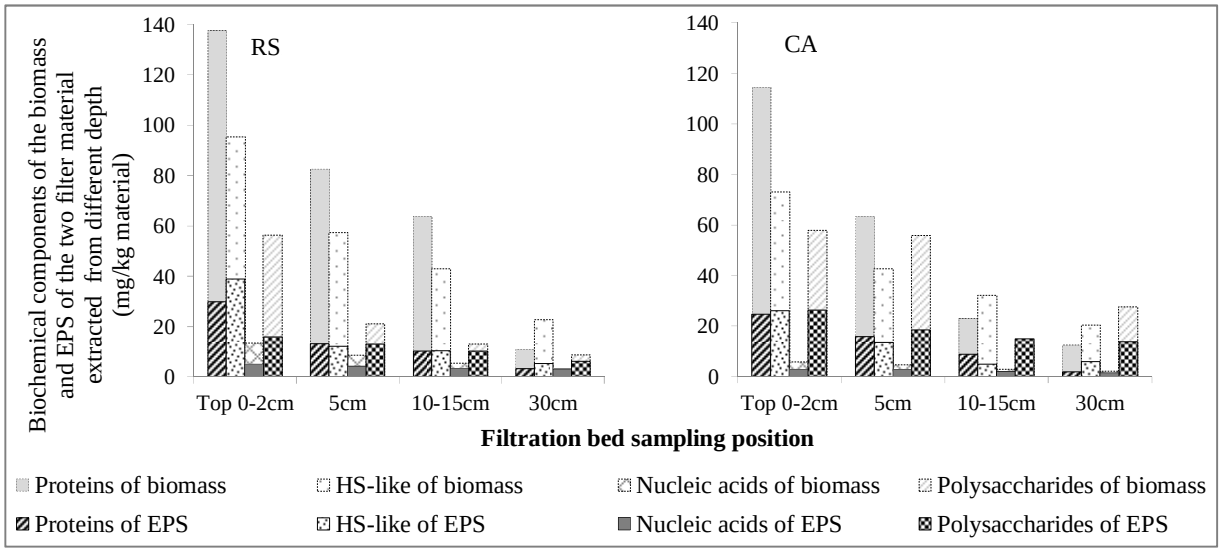

Figure 9: Biochemical composition of the biomass and EPS extracted from RS and CA in different filtration bed layers

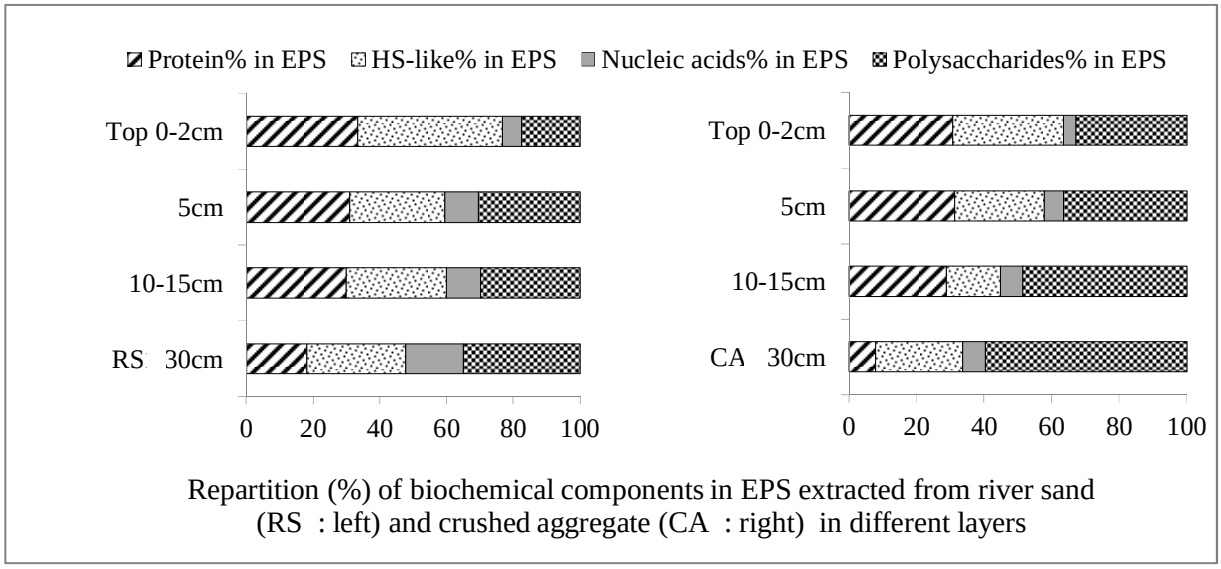

Figure 10 : Biochemical component proportions of the EPS extracted from RS and CA in different filtration bed layers

2. Vertical evolution of proteins and HS-like fingerprints:

The MW evolution of PN-like compounds in the 10-15 cm and 30 cm depths was also studied by means of HPSEC fingerprints. These results, presented in S2 (supplementary data), show similar fractions of PN-like EPS to those in the top layer; moreover, the four major fractions were identified as in Figure 3. The corresponding fractional percentage calculations are shown in Fig. 11.

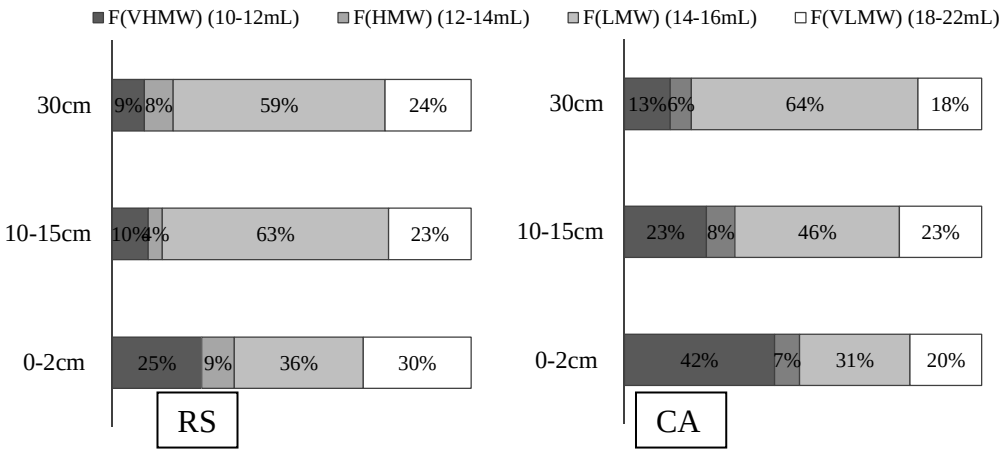

Figure 11: Percentage of areas of PN-like fingerprint fractions of the EPS, extracted from RS and CA over the different filtration bed depths

For both materials, the 10-15 cm and 30 cm layers exhibited a lower percentage of VHMW and HMW fractions yet a higher percentage of LMW and VLMW fractions, thus indicating that fewer high MW PN-like polymers were formed in the lower part of the filters. These low aMW compounds (<6kDa) may be amino acids-like or small peptides-like or molecules of similar configurations conveyed by the feed water, then transported and sorbed onto the deep medium. The LMW and VLMW fractions may also be associated with low MW PN-like molecules, resulting from the degradation of substrate by means of microorganism metabolism (Ni *et al*., 2011).

The HS-like fingerprint evolution over the depth of two materials has also been compared and summarized in Figure 12. These fingerprint chromatograms reveal similar shapes to the two filter materials and feed water, which suggests that HS-like compounds stem from the retention of exogenic organic matter. The HS-like fingerprints also display overlapping peaks with the 10-15 cm and 30 cm samples of both materials and moreover indicate that HS-like may be captured from the feed water and distributed relatively homogeneously inside the

filter medium, despite the fact that the types of filter materials and these exogenic HS-like compounds possess similar and low MW (< 6 kDa).

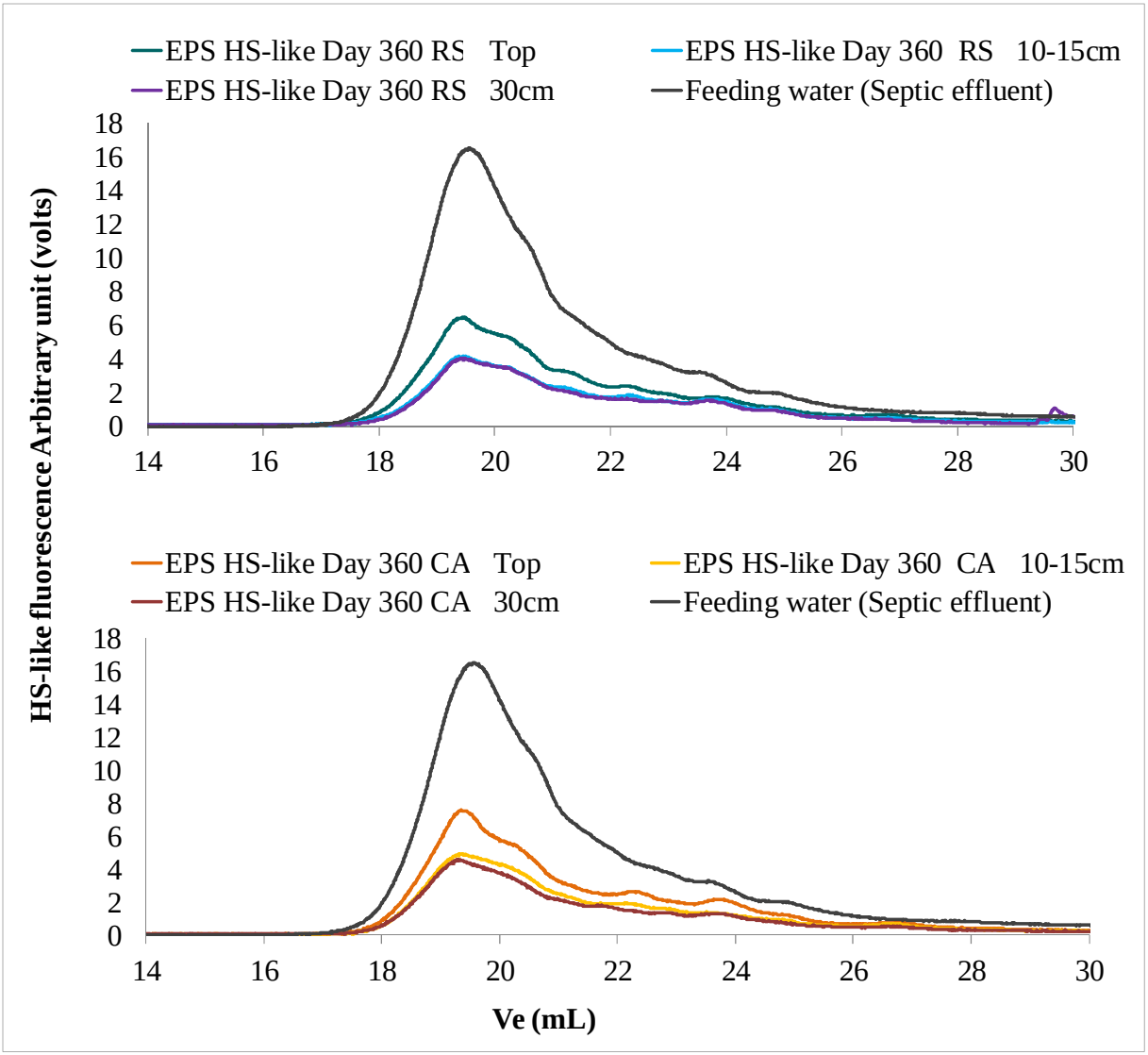

Figure 12: Evolution in HS-like fingerprints of extracted EPS and biomass over time in both river sand (RS) and crushed aggregate (CA)

4. Conclusion

This paper has sought to provide in-depth information regarding the EPS extracted from a biofilter at various enrichment stages during a 360-dayperiod and for different bed thicknesses. Two types of packing materials, river sand and crushed aggregate, were used and a series of quantitative and qualitative analysis conducted to investigate the differences in EPS characteristics. The following conclusions could be drawn:

Overall, the percentages of EPS in biomass increase with biomass enrichment despite a decrease due to humic-like substances as of Day 210. During biomass enrichment, the humic-

like substance/protein ratio decreases. Meanwhile, within the bed thickness, the polysaccharide/protein ratio rises by Day 360. Moreover, a correlation between the higher percentage of polysaccharide in EPS and less favorable environment for microorganisms potentially found in the CA biofilter has been proposed. The packing material mineral characteristic does affect polysaccharide implementation in EPS, in considering the divalent cations possibly released with CA.

A clear difference in the SEC protein-like fingerprints during biomass enrichment, in terms of both intensity and number of peaks, was noticed. This finding indicates that diverse molecular structures have occurred as a result of the enrichment; a variation in the four fractional distributions (i.e. very high, high, low and very low MW) was also shown. When decreasing, the high MW fraction can reveal bacterial growth and aggregation in the biofilm. An increase in the very high MW fraction may be correlated with bacterial aggregation. Within the bed depths, the very high MW fraction decreases, thus suggesting less aggregation. This fraction decreases after Day 210, but only for the RS material, which is perhaps connected to a more mature biofilm.

The SEC fingerprint applied with detection for HS-like also provided valuable information. It was shown that the HS-like from EPS have weak MW and result from the adsorption of feed water, perhaps initiated either by the preference for smaller humic molecules (aMW<720 Da) or by an HS-like molecule being metabolized by biofilm cells at the beginning of the process (up to 210 days). On Day 360, HS-like SEC fingerprints are the same as those from the feed water (aMW<6kDa) in the biofilter.

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Conclusion:

Les études montrent clairement que la matière organique du biofiltre n'est pas une accumulation de la matière organique de l'effluent mais bien une production par le biofilm (pour la biomasse totale et l'EPS). La teneur en protéines est ainsi beaucoup plus faible pour l'effluent ainsi que la fraction de protéine-like de forte masse moléculaire (MM).

Au cours de la mise en place du procédé, la biomasse totale et les EPS augmentent et en particulier les teneurs en protéines de la biomasse.

Les résultats montrent une augmentation plus forte de la biomasse pour RS1 mais aussi une stabilisation pour RS1. Pour le réacteur contenant CA1, le développement de la biomasse semble plus long et semble encore en pleine croissance à l'issu des 360 jours de procédé. Le % d'EPS augmente dans le temps avec une teneur légèrement plus importante tout le long du procédé pour CA1. La répartition protéine/sucre est différente en fonction du type de matériaux ; le ratio augmente au cours du temps pour RS1 alors qu'il diminue pour CA1.

L'étude par une méthode de chromatographie d'exclusion stéarique tend à démontrer que les substances humiques présentes majoritairement dans la biomasse et l'EPS seraient issues d'une accumulation de l'effluent au niveau de la matrice extracellulaire, bien que des empreintes de substance humique-like soient identiques entre l'effluent et les EPS après 210 jours de fonctionnement.

Les empreintes SEC des protéines-like de la biomasse montrent au bout de 210 jours une tendance vers une augmentation des fractions de fortes masses molaires (MM) même si la répartition est un peu différente pour RS1 et les CA. Pour les EPS, l'évolution des fractions des protéines like est par contre opposée : alors que la fraction de très haute MM tend à diminuer au cours du temps pour RS1, cette fraction augment pour CA1.

A 360 jours le rapport protéines/sucres diminue en fonction de la profondeur du biofilm et ce d'autant plus pour le biofiltre contenant CA1. La fraction de forte MM des protéines like tend à diminuer avec la profondeur dans le biofiltre et ceci pour les deux types de matériaux.

Ces résultats montrent que la caractérisation de la matière organique de la biomasse et des EPS est un critère potentiel de choix pour différencier les deux types de matériaux dans les biofiltres, mais plus d'études sont nécessaires. Les futures études devront notamment se

focaliser sur les protéines et le rapport protéines/sucre ainsi que sur l'évolution au cours du temps des empreintes SEC des protéines-like.

Part III: Conclusion générale

Dans les systèmes d'assainissement autonome, une étape de filtration biologique intervient après le traitement anaérobie de l'effluent. L'environnement du lit filtrant avec ses conditions de milieu insaturé est un milieu complexe et hétérogène qu'il est difficile d'appréhender. Des mécanismes très différents permettent d'éliminer des compartiments très différents de la contamination des eaux usées : filtration physique des solides en suspension et de microorganismes, précipitation chimique de minéraux, dégradation biologique et conversion de contaminants du carbone et de l'azote. Les processus biologiques sont le moins bien connus car ils impliquent aussi bien l'assimilation/dégradation de la matière organique que le développement de la biomasse/biofilm.

Le sable de rivière est généralement utilisé comme matériau de garnissage et comme support de biomasse dans les filtres. L'exploitation importante du sable de rivière rend la ressource fragile et nécessite la recherche de matériaux de substitution. Les agrégats concassés disponibles et économiquement avantageux sont envisagés en substitution du sable. Quelques études ont montré la possibilité d'utiliser des supports concassés comme matériaux de garnissage dans les lits filtrants mais les conséquences de cette substitution sur l'épuration des eaux ne semblent pas bien maitrisées. Une étude des impacts de la nature des matériaux de garnissage et de leurs caractéristiques physiques et chimiques sur le fonctionnement des filtres est donc nécessaire. Notre approche a consisté à rechercher des éléments de différenciation du comportement des filtres. En plus de l'efficacité épuratoire des filtres sur le carbone, l'azote et le phosphore, le développement et la stabilisation de la quantité et de la qualité du biofilm ont été suivis au cours du temps. Une étude du fonctionnement de différents filtres sur une longue période (360 jours) a été adoptée afin d'observer des différences de comportement de la phase de colonisation à l'état stationnaire. Le travail a consisté à caractériser au mieux les matériaux, puis à observer l'impact des matériaux sur les différents rendements et sur le développement de la biomasse avec un focus particulier sur les substances polymériques extracellulaires. Une interprétation du fonctionnement des filtres en fonction de la nature des matériaux est proposée.

III.1. Différentiation des matériaux

Une méthodologie concernant la mise en œuvre de matériaux filtrants et une caractérisation de ces matériaux granulaires ont été proposées :

Définition d'un procès expérimental :

Pour répondre aux objectifs, la première étape a consisté à définir et à construire un dispositif expérimental à l'échelle du laboratoire. À cet effet, le pilote de filtration a été composé de 12 réacteurs de filtration de 30 cm de diamètre et de 3 hauteurs différentes de garnissage : 15, 30 et 70 cm. La hauteur de 70cm étant la hauteur de matériau recommandée par la norme NF, DTU 64.1 (AFNOR, 2011) alors que les hauteurs de 15cm et 30cm sont considérés comme les couches actives du processus de filtration. Un aperçu de l'unité pilote de filtration est proposé sur la Figure 3.1.

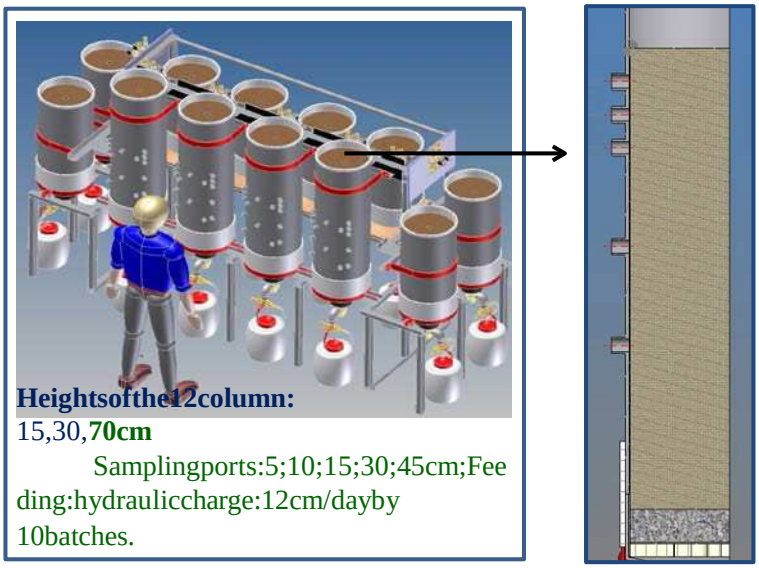


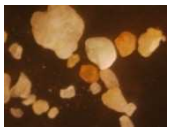
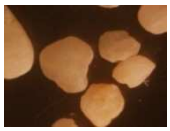
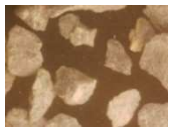
Figure 3.1:vue d'ensemble des pilotes de filtration

Caractérisation des matériaux de filtration :

Deux sables de rivière (RS1, RS2) et deux granulats concassés en carrière (CA1, CA2) ont été choisis pour cette étude. Des différences ont été observées dans la taille (analyse granulométrique), la forme de particules (analyse d'images) et la composition minéralogique (analyse chimique des lixiviats). Parmi les granulats concassés, CA1 a montré la plus forte

hétérogénéité dans la taille, avec un contenu de particules fines plus élevée et une surface spécifique plus faible (4.04 m^2/kg pour RS1 vs 2.78 m^2/kg pour CA1 et 2.88 m^2/kg pour CA2). Le sable RS1 est par sa taille le plus proche de la norme NFDTU64.1 et est considéré comme référence pour l'étude. Au contraire, RS2 peut être considéré comme un sable grossier et doit permettre de différencier l'effet de taille. CA2 ne présentait pas de caractéristiques extrêmes comme CA1, mais une minéralogie très hétérogène a été observée. Les principales caractéristiques de ces matériaux sont résumées dans le Tableau 3.1.

Table 3.1: Principales caractéristiques chimiques et physiques des matériaux de filtration choisis

Matériel	RS1	RS2	CA1	CA2
Nature	La Loire	La Loire	carrière	carrière
Photo de l'échantillon				
Effectivesize:D_{10}(mm)	0.38	1.60	0.17	0.44
diamètre moyen : D_m(mm)	0.82	2.26	1.36	1.6
Coefficient d'uniformité (D_{60}/D_{10})	2.78	1.75	10	5
Particule fines (<0.08mm)%	0.4%	0.5%	5%	2.4%
Rondeur (-)	0.74 (±0.11)	0.76 (±0.10)	0.73 (±0.15)	0.67 (±0.15)
Ca relargué (mg/kg)	4.30	2.28	10.43	28.72

III.2. Impact des matériaux sur le fonctionnement du réacteur : hydrodynamique et transfert de l'O_2

Plus le temps de séjour moyen dans les colonnes de filtration est important (HRT) (93h pour CA1 vs. 38h pour RS1) et plus le volume de rétention d'eau est fort (13% for CA1 and 8% for RS1). Cette rétention réduit également la conductivité hydraulique (coefficient de perméabilité) avec des valeurs très différenciées entre CA1 et RS1 (CA1: 3.25×10-4 m/s et

RS1: 8.95×10-4m/s). La présence de particules fines dans CA1 est donc susceptible d'impacter fortement le fonctionnement d'un filtre.

La mesure de la distribution de l'oxygène dans les deux filtres précédent a montré un niveau relativement constant d'O_2 (environ 19% v/v) et apparait dans tous les cas éloigné de la saturation. Pour RS1, l'introduction des bâchées entraine une baisse transitoire du taux d'oxygène (11 à 20%).

Les facteurs de forme très différents (rondeur par exemple) comme pour le matériau CA2 laisse présager par sa configuration anguleuse la présence de microenvironnements avec des chemins préférentiels de l'eau ou des transferts en air différent. La diminution de la conductivité hydraulique réduit l'infiltration et augmente les zones de saturation en eau. La Figure 3.2 propose une représentation conceptuelle de cette différence de comportement entre les deux classes de matériaux.

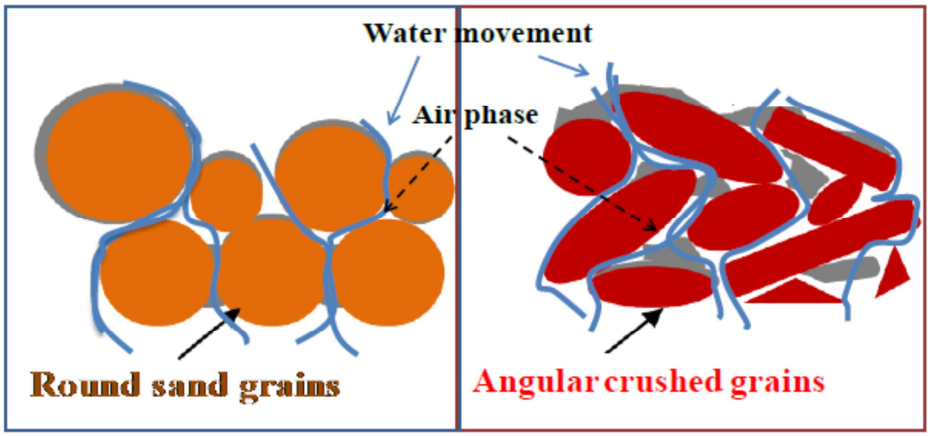


Figure 3.2: Proposition d'un schéma conceptuel reprenant les deux structures de grains (sable de rivière et agrégats concassés) et la distribution de l'air et de l'eau dans le lit.

III.3. Impact des matériaux sur l'épuration des eaux

L'objectif d'un réacteur de filtration dans le traitement in situ des eaux usées est de réduire autant que possible la quantité de matières solides, la matière organique, l'azote, les micro-organismes et éventuellement le phosphore. Des efficacités similaires ont été observées pour l'abattement des matières en suspension et de la matière carbonée par le sable fin de rivière (RS1) et par les deux granulats concassés (CA1, CA2).

Avec des tailles efficaces similaires, RS1 (matériel de référence) et CA1 (très hétérogène en taille avec de fines particules) ont montré des capacités de rétention mécanique identique vis-à-vis des particules et des matières organiques. Des différences entre ces deux matériaux sont par contre observées dans le suivi de l'élimination de l'azote total : l'élimination de l'azote par dénitrification est meilleure avec le sable fin de rivière. Comme nous l'avons vu précédemment, la distribution de l'oxygène dans le réacteur est influencée par la configuration du filtre : dans RS1, l'alternance entre les phases aérobie et anoxique pourrait être atteinte avec la finesse et l'homogénéité de la taille des grains. Le fluide dispersé dans le milieu pourrait chasser l'air au moment de l'alimentation du filtre ; dans CA1, en raison de l'hétérogénéité de la taille des grains, surtout de la présence des fines particules et de l'angularité des grains, des micro-environnements pourraient être différents par rapport au matériau de forme régulière. Cette hypothèse reprend le concept de microsites du sol proposé par Parkin (1987) et Gill *et al*., (2009). Le concept de micro-environnements pour les deux types de matériau est proposé sur la Figure 3.3.

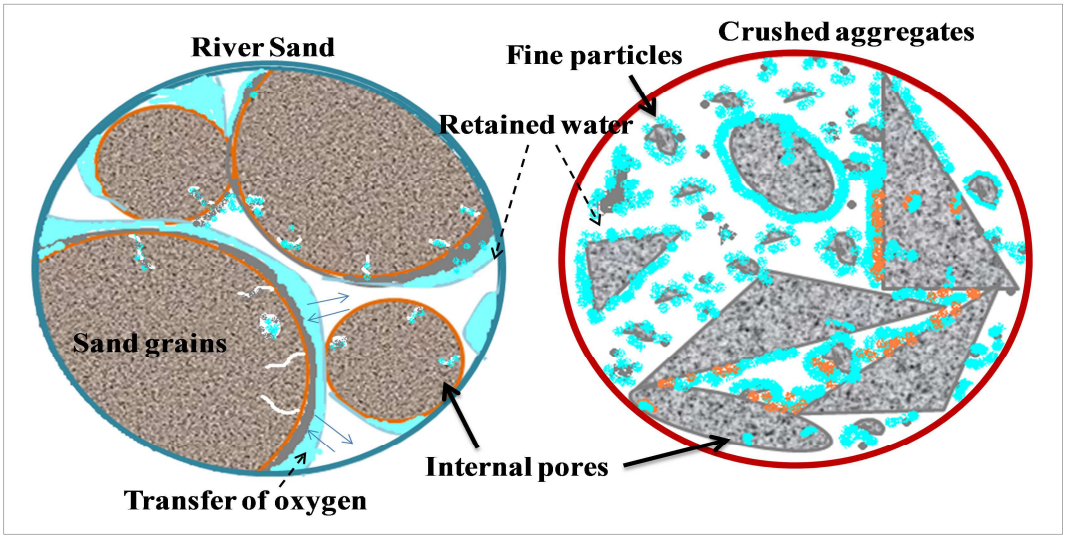


Figure 3.3: Concept de micro-environnements au sein des filtres garnis de sable ou d'agrégats concassés

L'évaluation de la biomasse a également indiqué, conformément aux attentes, une distribution verticale des différents types de bactéries avec la profondeur du lit de filtration. La distribution de la flore hétérotrophe et autotrophe semble être gouvernée par l'accessibilité des substrats et par la taille des grains. Par exemple, on observe que la flore hétérotrophe

diminue de façon significative avec la profondeur dans RS1 ($4,7 \times 10^7$ UFC/100 ml en haut et 5×10^5 UFC/100 ml à 30cm). La flore autotrophe est plutôt observée au niveau des couches de 10-15cm (6×10^3 et $1,8 \times 10^3$ UFC/100 ml pour RS1 et CA1) et 30cm (6×10^3 UFC/100 ml pour CA2).

Les rendements d'élimination en phosphate sont relativement instables dans les systèmes de filtration, cependant les granulats concassés montrent une meilleure élimination des phosphates. De plus, les rendements diminuent à moins de 60% après 120 jours de fonctionnement. La minéralogie du support qui a été estimée par lixiviation du support, pourrait expliquer cette élimination des phosphates par précipitation ainsi que le comportement des filtres dans le temps avec une diminution de la concentration en cations (Zanini *et al*., 1998).

Les résultats précédents montrent que des éléments majeurs peuvent impacter les rendements épuratoires sur des filtres biologiques: la taille des grains (moyenne et distribution) est un paramètre essentiel dans le choix des matériaux de garnissage; la présence de particules fines dans les granulats concassés est un facteur défavorable au bon fonctionnement du filtre.

Si les premiers bilans effectués sur les rendements épuratoires ont montré un comportement différent selon les matériaux, une meilleure compréhension de l'impact de la nature des matériaux notamment les agrégats concassés s'est avérée nécessaire. Nous nous sommes donc intéressés au comportement interne des réacteurs avec une approche quantitative et qualitative concernant la biomasse/ biofilm et les EPS. Pour cela, deux filtres respectivement garnis avec le sable de rivière RS1 et de l'agrégat écrasé CA1 ont été comparés pour des conditions d'effluent standard et avec une charge volumique 12cm/jour. Ces conditions ont été choisies car les rendements épuratoires sur les deux matériaux apparaissaient comparables pour des caractéristiques de matériaux très différentes. CA2 a été utilisé comme matériel supplémentaire en raison de ses caractéristiques intermédiaires entre RS1 et CA1.

III.4. Biomasse et matériaux du massif filtrant

La biomasse développée sur les couches supérieures des deux types de matériaux (RS1 et CA1, CA2) a été suivie en termes de quantités et de caractéristiques. L'évolution du rapport protéines (PN) /polysaccharides (PS) (les protéines étant considérées comme un indicateur de biomasse récente), l'évolution des masses moléculaires des protéines (ou assimilées – like en

anglais) par HPSEC couplée à une détection par spectrofluorescence. En comparaison avec la qualité de l'eau d'alimentation, la biomasse totale du filtre montre à travers les différents indicateurs une augmentation de la biomasse récente: augmentation des protéines (à la fois la quantité et les proportions de la biomasse) et du rapport PN/PS ; via les chromatogrammes de HPSEC, l'augmentation de la fraction des composés protéiniques (PN-like) de haute PM (> 1000kDa) est aussi observée. Les différents matériaux ne semble pas modifier la nature de la biomasse, mais par contre modifie les quantités totales de biomasse (plus faibles quantités de biomasse avec les granulats concassés) ainsi que le temps de stabilisation (pas de stabilisation en fin de l'étude dans les colonnes garnies de granulats concassés).

Il semble que la surface spécifique joue un rôle important dans l'établissement de la biomasse et une surface spécifique plus faible conduirait à un ralentissement de la production de biomasse. Plus le temps de séjour moyen et la porosité sont importants et plus l'organisation structurelle de la biomasse sera modifiée.

La biomasse totale (biofilm) comprend non seulement des cellules bactériennes avec leurs composants intracellulaires, mais aussi la matrice extracellulaire qui contribue à l'organisation structurelle du biofilm. Ainsi, l'étude ciblée des EPS peut compléter cette première approche avec une production et une composition en EPS peut être plus sensible à la nature de l'environnement du biofilm.

III.5. Evolution des EPS en fonction des matériaux de garnissage

EPS du matériau de référence, RS1 et effluent :

Le pourcentage d'EPS augmente en fonction du temps du procédé: RS1: 9~30%. Les protéines extracellulaires augmentent aussi entre le jour 60 et le jour 360 (<20mg/kg à 60mg/kg), et représentent environ 40% des EPS. Au jour 360, les empreintes HPSEC des protéines-like montrent des évolutions similaires entre la biomasse et les EPS et se différencient progressivement des empreintes SEC de l'effluent via la diminution de la fraction de faible masse moléculaire (MM apparente = MMa<6kDa) et l'augmentation de la fraction de très forte masse (>1000kDa). De 60 à 210 jours la proportion de forte MM augmente de 30% à 40% sur l'empreinte SEC de protéine-like. Ces résultats confirment que la matière organique présente dans le biofiltre n'est pas liée à une accumulation d'effluent mais en partie au moins à une production microbienne. Les HS-like (substance humiques-

like) seraient d'origine exogène à l'EPS. Or, même si au jour 250 la majorité des HS-like présente une MM légèrement inférieure à celle de l'effluent (Mma<6000Da) leur empreinte SEC est identique à l'empreinte HPSEC des substances humiques (HS-like) de l'effluent au jour 360. Ces résultats confirmeraient que les substances humiques des EPS sont des molécules venant de l'environnement dans le cas d'un procédé biofiltre.

Comparaison des EPS pour différents temps de procédé en fonction du matériau :

Certaines évolutions de la matière organique des EPS sont similaires entre RS1 et CA1, ainsi, la proportion de protéines augmente au cours du procédé (20% au jour 60 et 30% au jour 360). Les empreintes SEC des protéines-like ont des évolutions similaires entre les deux matériaux mais la fraction de très forte MM apparait moins rapidement pour CA1 comparé à RS1. La biomasse est moins présente et moins active (moins de protéine dans la biomasse totale) dans le réacteur avec CA1. Ce déficit serait généré par l'environnement moins homogène et la présence de zones de courts-circuits dont l'origine serait la teneur plus importante en particules fines (Figure 3.3). Cet environnement pourrait induire un stress responsable d'une plus forte production d'EPS soit par une flore microbienne similaire à celle trouvé dans RS1, soit parce que ces nouvelles conditions induisent un changement de communautés microbiennes, à l'origine d'un autre type d'EPS. Le pourcentage d'EPS dans le CA1 augmente en effet légèrement par rapport à RS1 au cours du temps (20 à 32% aux jours 60 et 360 pour CA1). Les EPS présentent aussi une plus forte proportion en polysaccharides avec CA1 (18 à 33% pour CA1 contre 30 à 17 pour RS1 du jour 60 au jour 360). Les SH-like ne sont pas impactées par le type de matériau. La minéralogie du support peut aussi influencer les caractéristiques des surfaces des grains qui intervient dans le mécanisme d'adhésion des bactéries (Rose et *al.*, 1993), ou spécifiquement avec les EPS (Higgins & Novak, 1997). Les cations divalents relargués (Ca^{2+}) par les agrégats concassés sont peut être à l'origine de la variation de la composition biochimique des EPS. Le Ca^{2+} permet ainsi de former des complexes appelés « boîte à œuf » qui sont composés de polysaccharides et de Ca^{2+} (Sobeck & Higgins 2002).

Comparaison des EPS en fonction de la profondeur du réacteur en fonction du matériau :

La concentration en substrat de l'effluent diminue avec la profondeur puisque les substrats commencent à être dégradés dès la couche superficielle du biofiltre. La distribution de la biomasse totale correspond au profil décroissant en disponibilité de substrats pour les deux

matériaux. La masse d'EPS diminue aussi mais une proportion plus importante d'EPS est observée dans les couches profonde (RS1: 30% au sommet vs. 39% à 30cm et CA1: 32% vs. 37%). Le stress généré par la diminution de substrat disponible en profondeur peut expliquer l'augmentation de la proportion en EPS, de même que le changement de la flore microbienne. En Effet, en profondeur, les bactéries nitrifiantes autotrophes sont plus favorisées que les autotrophes des couches superficielles, la nature et la quantité des EPS produits par une autre communauté microbienne peuvent alors varier.

III.6. Conclusion technique

L'étude de la matières organique du biofilm et en particulier celle de l'EPS a permis de différencier clairement deux matériaux (RS1 et CA1) qui avaient pourtant montré sur 360 jours de procédé des capacités épuratoire similaires. Le suivi, en particulier dans le temps des protéines (MM) ou plus simplement de rapport Protéines/Polysaccharide des EPS semble assez pertinent pour différencier les matériaux.

Cette étude dans ses aspects techniques a permis une meilleure compréhension du rôle des matériaux de garnissage dans des réacteurs de filtration insaturés à travers deux aspects dépendants l'un de l'autre: l'efficacité d'épuration et les évolutions de la biomasse/des biofilms avec le temps et la profondeur. Les principaux facteurs d'influence qui ont été mis en évidence sont :

- la distribution granulométrique: la taille des grains est le principal paramètre qui régule l'élimination de la matière particulaire dans l'effluent septique, y compris les minéraux et les particules organiques. Une granulométrie fine augmente également la colonisation de la couche supérieure des lits de filtration par la biomasse et accélère l'obtention d'un état stationnaire avec une faible production d'EPS, en particulier en polysaccharides. Cette influence est observée dans le cas d'un sable de rivière fin (RS1) (D_{10}=0,38 mm);
- la taille moyenne des grains: une taille moyenne importante comme le RS2 (Dm > 2.0 mm) réduit la surface spécifique et doit être évitée en raison de la réduction trop importante du mécanisme de filtration mécanique. L'hétérogénéité plus élevé observée avec les granulats concassés réduits la taille moyenne calculée mais avec un pourcentage de grosse particule non négligeable. La présence des petites particules rend l'environnement de filtration plus compliqué et limite l'interprétation de l'effet

de la taille moyenne (canaux d'écoulement tortueux ou chemin préférentiel des phases air ou liquide);

- le facteur de forme des particules: comme supposé par le concept proposé sur la Figure 3.2, les formes des particules doivent jouer un rôle important sur le comportement de la phase liquide et l'hydrodynamique du système, ainsi que sur la distribution de la phase gazeuse. La distribution inégale des substrats ou de l'air peut créer une accumulation hétérogène de la biomasse et des obturations locales du lit;
- les particules fines : comme proposé sur la Figure 3.3, la présence de particules fines est un paramètre majeur qui influence les propriétés hydrauliques et hydrodynamiques des lits de filtration. Les particules fines jouent un rôle "d'éponge" en créant un milieu favorable au développement du biofilm) mais réduit également le taux d'infiltration et provoque un risque de colmatage;
- la composition minéralogique: elle est impliquée dans le processus d'élimination des anions (tels que les phosphates) par production de cations complexants. Elle pourrait influencer la mise en place du biofilm.

Ainsi, dans le cadre de l'intérêt croissant pour les granulats concassés, cette étude comparative pendant 360 jours des rendements épuratoires et du développement du biofilm entre des réacteurs de filtration garnis de granulats concassés et de sables de rivière, apporte des éléments essentiels concernant les limites à la substitution des sables. Ce travail a contribué à la compréhension fondamentale accrue sur les mécanismes de filtration et en particulier sur l'impact des matériaux de filtration sur le processus de développement de la biomasse / des biofilms. Il permet de valider des notions de taille moyenne nécessaire au compromis entre rétention mécanique, développement de biofilm. Il met en évidence les risques associés à une distribution trop importante de la taille des grains et les difficultés de prévision de fonctionnement en présence de fines (et donc éventuellement de matériaux friables).

III.7. Perspectives

Les matériaux de garnissage doivent être parfaitement étudiés avant leur utilisation et des recherches seront toujours nécessaires notamment sur la production d'EPS et les éléments colmatant comme les polysaccharides. Pour comprendre la formation et la distribution du biofilm sur les différents matériaux, des études supplémentaires doivent être menées afin de

parfaitement maitriser le comportement des filtres sur le long terme (5 à 10 années) avec notamment les aspects :

- Microbiologique: l'étude de la répartition des communautés bactériennes et de leur diversité au sein et dans la profondeur des lits de filtration est nécessaire afin de parfaitement appréhender la production des différents constituants EPS;
- Biochimique: en considérant les polysaccharides extracellulaires comme un indicateur du risque de colmatage une étude portant sur la caractérisation plus précise de ce groupe d'EPS peut aider à mieux comprendre le colmatage potentiel des différents matériaux et permettrait de mieux répondre aux facteurs d'influence qui gouvernent l'excrétion;
- Hydrodynamique: dans notre étude, différents arrangements entre les particules minérales ont été proposés avec un comportement adapté à chacune de ces configurations (Figure 3.2). Des études plus approfondies doivent être réalisées pour décrire les mécanismes et l'hydrodynamique à l'aide de traceurs spécifiques (tels que des traceurs radioactifs ou colorant) et valider cette proposition.

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Résumé français :

L'assainissement non collectif concerne 12 à 15 millions de personnes en France. La filière classique de ce mode d'assainissement se compose généralement d'un prétraitement anaérobie par une fosse septique recevant l'ensemble des eaux usées domestiques suivi d'un système d'infiltration dans le sol ou d'un filtre à sable. Le filtre à sable vertical drainé met à profit le pourvoir épuratoire qui est principalement lié à la présence d'une biomasse sous forme d'un biofilm. Cette dynamique de la croissance de la biomasse ou du biofilm est soumise à l'impact de la nature de matériaux filtrants. L'écoulement insaturé dans ces systèmes conditionne également cette croissance du biofilm.

Dans ce contexte, l'objectif du travail de la thèse est d'appréhender les mécanismes mis en jeu et particulièrement l'impact des matériaux dans le fonctionnement des filtres en comparant notamment deux types de matériaux: les sables de rivière et les agrégats concassés. Pour cela, une étude expérimental sur une unité pilote composé des réacteurs de filtration du diamètre de 30cm et différents épaisseurs de garnissage (15, 30 et 70cm) a été construite. Les réacteurs garnis de deux sables roulés et deux agrégats concassés, sont alimentés en effluent septique avec une charge volumique 12cm/jour par 10 bâchés par jour. Suite des matériaux, une étude de la performance épuratoire avec le suivi des composants biochimiques de la biomasse totale et de la matrice extracellulaire du biofilm est réalisée en comparant notamment les deux types de matériaux filtrants.

L'étude des matériaux et des propriétés des réacteurs filtrants ont montré que les agrégats concassés présentaient une hétérogénéité des tailles et des formes des grains, ainsi qu'une teneur élevée en particules fines (<0.08mm). L'hydrodynamique dans le réacteur garnis avec l'agrégat concassé possédant la teneur en particules fines le plus élevée est caractérisé par un temps de séjour moyen plus important. A la différence du sable roulé qui forme un environnement très uniforme, les agrégats concassés avec la présence des particules fines et des grains très anguleux peuvent créer des microenvironnements avec de plus forte turbulences, ou inversement des endroits peu accessibles à l'air ou au liquide.

La capacité épuratoire des matériaux filtrants est gouvernée principalement par la taille des grains, surtout pour la pollution particulaires et organiques, ainsi que pour l'élimination des ions ammoniacs. Un meilleur rendement de l'élimination de l'azote total est observé dans le réacteur garnis de 70cm du sable roulé fin, sous la charge hydraulique 12cm/jour. Une alternance de la phase aérobie/anoxique apportée par les bâchés dans ce fin médium favorise la dénitrification. L'effet des microenvironnements provoqué par l'agrégat concassé dans un massif filtrant diminue également la dénitrification. Une augmentation de la charge hydraulique réduit cette condition par une diminution en temps de séjours.

Les évolutions de la biomasse totale et de la matrice extracellulaire se différentient également entre le sable roulé et les agrégats concassés lors du suivie des composants biochimiques (protéines, humic-like substances, polysaccharides and acides nucléiques) et les empreintes HPSEC des protéines-like. Le sable roulé fin a présenté une stabilisation de la croissance en biomasse totale le plus tôt avec une production des composants extracellulaires par unité de la biomasse moins forte que les agrégats concassés. La teneur en particules fines dans les agrégats concassés peut-être à l'origine de microenvironnements pauvres en substrat ou en oxygène, ou des différentes propriétés de surface de grains impactées par la composition minéralogique modifiant la production des composants extracellulaires, surtout les polysaccharides.

L'impact des différents matériaux filtrants est principalement lié à la taille de grains qui gouverne la rétention mécanique des polluants et en même temps gère l'établissement de la biomasse. L'effet des particules fines dans l'agrégat concassé en réduisant le temps de séjour et en créant des microenvironnements hétérogènes, peut provoquer le colmatage du filtre sur le long terme. De plus, les agrégats concassés ont montré des formes des grains anguleuses et irrégulières, avec pour conséquence un comportement hydrodynamique moins homogène et ramener une distribution en nutriment et en air non uniforme dans la massive. Ainsi, l'évolution de la biomasse et surtout la composition en exsudats extracellulaires sont particulièrement différenciée entre un sable roulé et un agrégat concassé. La composition minéralogique d'agrégats impacte l'élimination des phosphates et modifie également l'excrétion des composants extracellulaires: la proportion des polysaccharides dans les EPS est plus élevés dans l'agrégat concassé qui montre une composition plus riche en calcium.

Cette étude, en comparant deux types de matériaux de nature très différente, contribue par deux aspects à une meilleure compréhension du fonctionnement des réacteurs de filtration: le pouvoir épuratoire et la composition biochimique des composants de la biomasse totale et de la matrice extracellulaire. De cette étude, des éléments techniques peuvent être retirés pour l'utilisation alternative de matériaux non traditionnels. De bonnes conditions de fonctionnement peuvent être attendues avec une granulométrie similaire à celle des sables roulé d'une taille effective inférieure à 0.4mm et un coefficient d'uniformité le plus faible possible.

Des études plus spécifiques sur une période de temps plus longue permettraient d'approfondir les connaissances sur certains aspects : par exemple, la distribution des communautés bactériennes dans le temps et dans la profondeur des réacteurs, la répartition des phases et l'hydrodynamique du liquide et de l'air à l'intérieure du massif filtrant de grains anguleux avec l'utilisation de traceurs. L'intérêt d'une étude sur une échelle de temps très longue est la compréhension des dysfonctionnements d'un filtre réel, par exemple, le colmatage.