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Master's Thesis in the Master's Programme Infrastructure and Environmental Engineering, Nordic Master in Environmental Engineering

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ABSTRACT

Soluble microbial products (SMPs) are the pool of organic compounds originated from bacterial metabolism and decay and they constitute the major fraction of the soluble organic carbon in the effluent from biological treatment processes. SMP occurrence and characteristics are of a great significance with respect to discharge quality and performance of wastewater treatment processes.

In this study, two sets of aerobic batch reactors were used to investigate the growth, the organic carbon uptake and the release of SMPs by communities of bacteria fed with different volatile fatty acids (VFAs) as carbon sources (formate, acetate, propionate, butyrate). For each reactor, biomass growth, VFA biodegradation and SMP production as dissolved organic carbon (DOC) were examined. The examination of molecular weight (MW) distribution, analyzed with high performance size exclusion chromatography (HPSEC), and spectroscopic parameters (absorbance spectrum, specific ultraviolet absorbance (SUVA) and slope ratio (S_R) allowed understanding the pattern in the production of different types of SMP. The results revealed that the investigated reactors produced SMPs in different concentrations and types, and they were characterized by SUVA values <2 mg/L, reflecting hydrophilic characteristics. The molecular weight (MW) distribution of the DOC varied from very low (<100 Da) to high (>20 kDa). The calculated S_R values revealed to be a good proxy for SMP MW indicating a shift from low MW compounds to high MW along the course of the experiments, in accordance with HPSEC results.

Parallel to this investigation, an attempt to isolate bacteriophages was carried out in order to evaluate their impact on SMP production and to quantify their number in different stages of a wastewater treatment plant. Bacteriophages, or phages, are viruses that infect bacteria causing their lysis. They appear to be active components of activated sludge communities and the most numerous entities. The double layer plaque protocol was followed to isolated phages from activated sludge process treating urban sewage. Bacterial isolates from the batch reactors were used as hosts. None of the bacterial isolates supported plaque formation, suggesting that none of the strains were host for the phages present in the activated sludge reactor. The lack of plaques prevented culture counts. Enumeration of virus-like particle in a wastewater treatment plant was also carried out using a tunable resistive pulse sensing technique. The results showed counts of 10^9 to 10^{10} virus like particles, with higher abundance in the influent and activated

sludge reactor compared to the effluent. It was estimated that the virus like particles could make up as much as 6-20% of the DOC in the effluent from the sampled wastewater treatment plant.

KEY WORDS: soluble microbial products, aerobic batch reactors, volatile fatty acids, molecular size distribution, bacteriophages.

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CONTENTS

List of abbreviations

AS	Activated Sludge
BAP	Biomass Associated Product
DOC	Dissolved Organic Carbon
EPS	Extracellular Polymeric Substances
HPLC	High Performance Liquid Chromatography
HPSEC	High Performance Size Exclusion Chromatography
MBBR	Moving Bed Biofilm Reactor
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MW	Molecular Weight
OD	Optical Density
S_R	Slope Ratio
SMP	Soluble Microbial Products
SUVA	Specific Ultraviolet Absorbance
TOC	Total Organic Carbon
UAP	Utilization Associated Products
VFA	Volatile Fatty Acid
VLP	Virus-like Particles
WWTP	Waste Water Treatment Plant
$\Delta G°$	Gibbs free energy

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1 INTRODUCTION

The activated sludge process is a widespread technique to treat municipal and industrial wastewater biologically. It exploits the natural capacity of complex microbial communities to remove pollutants from wastewater through oxidation of the organic load. There is still a lack of understanding about the interrelationships of different members in the activated sludge communities, and more work is needed to unravel the activities taking place in the microbial black box.

Soluble microbial products (SMP) is one factor that affects the quality of wastewater effluents and microbial activities in an unclear way. The organic carbon left in the effluents from a biological treatment process consists of a wide variety of soluble organic matter containing a small fraction of residual influent organic compounds and a larger fraction SMP, which are produced during bacterial metabolism and decay (Barker and Stuckey 1999; Laspidou and Rittmann 2002; Shin and Kang 2003). SMPs can affect the treatment performance, not just in terms of effluent quality, but they can also adversely affect the microbial community in terms of kinetic activity and settling characteristics because of their flocculating, chelating and toxic properties (Yan, Subramanian et al. 2007). SMPs may in some cases be even more toxic than the original organic matter present in wastewater (Aquino and Stuckey 2002). Therefore, the majority of the studies about these compounds have been directed to identify the SMP species, to quantify their production, to evaluate the most efficient posttreatment processes for their removal, and to assess the ways of reducing their production through control of process parameters. Most of the work has been done on aerobic and anaerobic systems with pure culture and/or defined feed and making use of this knowledge in a real system still an uncompleted task. A better understanding of the dynamics can lead to an optimization of the activated sludge process by minimizing SMPs and consequently the residual chemical oxygen demand in the effluent. The knowledge about SMPs is far from complete and more research needs to be directed to entirely understand their influence on the treatment performance.

Another factor that may affect the performance of the activated sludge process is predation by bacteriophages (phages). Phages, viruses infecting bacteria, are considered the most numerous and diverse entities on our planet, with a ratio of phages to bacterial cells of 10:1 in most of the studied ecosystem, including wastewater treatment plant (WWTP) (Shapiro and Kushmaro 2011). Since they are the most abundant biological entities in WWTP, it is believed that they play a central role in the ecology of activated sludge. Most of the work in this field has been focused on the detection of virus-like particles (VLP) as indicators of pathogenic viruses in the wastewater effluent (Baker and Herson 1999). Little is known about bacteriophage activity on the microbial ecology of activated sludge, but it has the potential to improve the performance and effluent quality, and to control some of the process problems by regulating key bacterial functional groups (Withey, Cartmell et al. 2005). However, before using phages to manipulate and control environmental biotechnology processes, a better understanding of phage interactions with their hosts is needed. It is also possible that phage activity

has an effect on the characteristics of SMPs, since it is expected that a bacterial cell lysed due to phage infection will release a range of SMP into the surrounding water. Phage particles may themselves also contribute to the SMP concentration in the water.

1.1 AIM

The main goal of this study is to investigate release of organic compounds by microorganisms in activated sludge. The specific objectives of this work are:

i. To investigate the growth, the organic carbon uptake and the release of soluble microbial products (SMPs) by communities of bacteria fed with different carbon sources.
ii. To investigate spectrophotometric methods for the characterization of SMPs.
iii. To quantify the number of bacteriophages in different stages of a wastewater treatment plant
iv. To isolate bacteria and bacteriophages and investigate the effect of their interactions on SMP production.

2 LITERATURE REVIEW

In this chapter, general background information are presented in order to provide the reader a foundation for the data analysis.

2.1 ACTIVATED SLUDGE

Activated sludge processes have been exploited for domestic and industrial wastewater treatment since the last century (Ramalho 2012). The process consists of the oxidation of soluble and particulate organic material, which serve as substrate for bacterial growth, with oxygen or nitrite and nitrate driven by a flocculent slurry of microorganisms, resulting in production of gaseous products and additional biomass. The reactor in which the process occurs is aerated in order to provide mixing and oxygen transfer. The solid fraction, which includes the biomass, is separated from the effluent and recirculated in the system. The excess sludge is removed from the system in order to maintain a desired solids retention time (Jenkins and Wanner 2014). The domestic wastewater provides the inoculum, the biological nourishment and the nutrients necessary for the microbial growth (Viessman, Hammer et al. 2009).

The aims of the activated sludge process are to reach high removal of nutrients and organic compounds within a short time and to produce flocs that are easy to settle and separate from the effluent (Wang, Pereira et al. 2009).

2.1.1 Characteristic and properties of the biological flocs

Activated sludge consists of several constituents such as growing microorganisms, extracellular polymeric substances (EPS), organic and inorganic matter, suspended and precipitated. The floc structure and properties are dependent on these constituents together with other factors, i.e. the physicochemical characteristic of the inflow (Nielsen, Thomsen et al. 2004).

The microbial community in activated sludge is very diverse. The *bacterial fraction* dominates and is constituted mainly by aerobic heterotrophic bacteria. Heterotrophic organisms use organic compounds as energy source and carbon source for synthesis of new biomass (Viessman, Hammer et al. 2009). The presence of lithoautotropic bacteria, which use CO_2 as carbon source and oxidize an inorganic compound as energy source, is also to be expected, such as nitrifying bacteria. *Fungi* may be present at low pH, which is an uncommon characteristic for domestic wastewater treatment reactors (Seviour, Blackall et al. 1999). *Algae* are microscopic photosynthetic plants, therefore they thrive in presence of light, which may be relevant in open systems (Seviour, Blackall et al. 1999). *Protozoans* are single-celled animals ingesting solid organic matter, including bacteria and algae, for energy and carbon source. *Metazoans* are multicelled animals that cope if the sludge age is high enough and feed on protozoans, bacteria and solid organic matter. The flocs of activated sludge include most of the important groups of microorganisms. Metazoans and protozoans, which continuously

graze on the activated sludge flocs, are not part of them since they can break away from them (Wang, Pereira et al. 2009).

Bacteria are the key player with the regards to floc characteristics and properties (Nielsen, Thomsen et al. 2004). Therefore, the operating condition of the WWTP are strongly dependent on the microbial community of the activated sludge. The understanding of the role of the organisms in the activated sludge ecosystem leads to a better control of the operation and to an improved performance (Nielsen, Thomsen et al. 2004). Single celled, floc forming or filamentous bacteria compose the bacterial biocoenosis of activated sludge. All three types degrade organics.

2.1.2 Principle of biological oxidation

Bacteria take in nutrients and substrates and convert them into energy for their metabolism, cell component and waste. The formula, which describes the complete oxidation of organics by activated sludge bacteria in an aerated tank assuming a period of infinite aeration, can be expressed as:

$$\text{Organics (C, H, O)} + \text{biomass} + O_2 \rightarrow CO_2 + H_2O + \text{biomass} + \text{energy} + \text{microbial products}$$

It has been observed that when the wastewater is mixed with activated sludge in an aeration tank a significant removal of organic matter occurs in the first few minutes depending on the characteristics of the incoming liquid and activated sludge. This process is interpreted as an adsorption phenomenon removing discrete and colloidal particles. The adsorbed organic matter is subsequently oxidized or used in the synthesis of cellular components (Wang, Pereira et al. 2009).

2.2 Soluble Microbial Products

Most bacteria produce three dissimilar microbial products: extracellular polymeric substances (EPS), soluble microbial products (SMP) and inert biomass (Laspidou and Rittmann 2002). EPS is a matrix of large polymeric molecules, the most abundant components being polysaccharides, proteins and nucleic acids, and it constitutes the microbial biofilm or the floc in a suspended culture (Laspidou and Rittmann 2002). EPS carries out several important functions, i.e. aggregation of bacterial cells, adhesion of microorganisms to a surface, protection against harmful external factors, retention of water, concentration of organic matter and nutrients, accumulation of extracellular enzymes (Laspidou and Rittmann 2002). Inert biomass is the part of the decayed active biomass that has not been oxidized to generate energy for maintenance needs (Laspidou and Rittmann 2002).

SMPs are defined as the group of soluble organic compounds released during bacterial metabolism. SMPs can be classified in two typologies: biomass associated products (BAPs), which are related to endogenous respiration, i.e. cell lysis and decay, and utilization associated products (UAPs) associated with biomass growth and substrate consumption (Namkung and Rittmann 1986). The UAPs are produced at a rate

proportional to the rate of substrate uptake, while BAPs are produced at a rate proportional to the concentration of biomass (Barker and Stuckey 2001). Therefore, UAPs are expected to prevail in substrate-rich conditions, while BAPs are likely to be found under substrate deficiency (Jarusutthirak and Amy 2007). SMPs are formed by a complex pool of organic compounds, such as proteins, polysaccharides, humic substances, nucleic acids, organic acids, amino acids, DNA, lipids, other carbohydrates, etc. (Barker and Stuckey 1999). Small carbonaceous molecules derived from the original substrate mostly compose UAPs, while BAPs are macromolecules composed by both carbon and nitrogen (Urbain, Mobarry et al. 1998).

SMPs are produced during normal bacterial growth and metabolism. They are expelled also to create a concentration equilibrium across the cell membrane. Other factors can intensify their formation, such as starvation or unavailability of a required nutrient, sudden increase in concentration of a carbon or energy source, environmental stress e.g. osmotic shock and temperature changes (Barker and Stuckey 1999; Lebrun, Thieblin et al. 1999). The characteristics of SMPs are dependent on the microbial populations, the substrate and its utilization rate, and other physicochemical condition, such as pH, temperature, redox potential, retention time etc. (Barker and Stuckey 1999; Shin and Kang 2003).

An important parameter in their investigation is the molecular weight (MW) distribution. It has been showed that the distribution is very wide (from <0.5 kDa to >100 kDa), but is bimodal with a peak of distribution in the low molecular weight (LMW) region (<1 kDa) and a spike in the high molecular weight (HMW) region (>10 kDa). UAPs are mostly distributed in the LMW region, while BAPs are mostly composed of large molecules (Boero, Bowers et al. 1996).

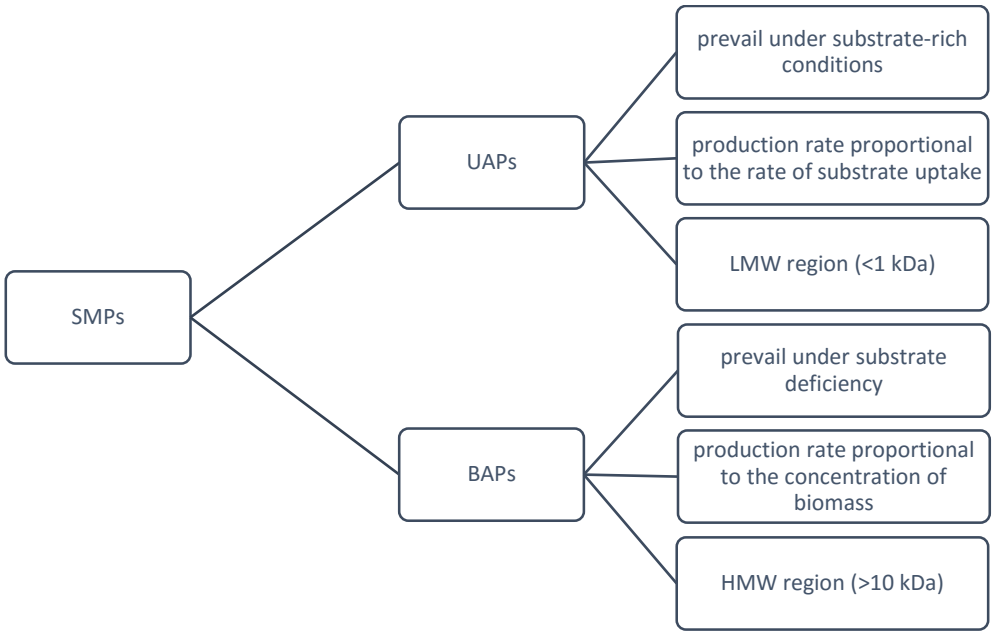

Figure 2.1: Fractions of SMPs

Most of the SMPs are biodegradable in both aerobic and anaerobic systems over a time that is usually longer than the conventional hydraulic retention time of the processes (Schiener, Nachaiyasit et al. 1998).

Most of the soluble organic matter in the effluent from a biological treatment process has been proven to be SMP (Barker and Stuckey 1999; Jarusutthirak and Amy 2007). The dissolved organic carbon (DOC) effluent from a biological process is composed by the fraction of untreated DOC coming from the influent and the SMP formed during the treatment (Lebrun, Thieblin et al. 1999). SMPs that remain in the effluent from the wastewater process represent the upper limit of treatment performances, reducing the efficiency of the system (Yan, Subramanian et al. 2007). Besides this issue, it has been shown that membrane internal fouling is mainly due to these soluble microbial products (Fonseca, Summers et al. 2007)

2.2.1 Analytical methods for SMPs

The identification of SMPs is challenging, since multiple unknown compounds compose them and they do not belong to a specific group. As there is no standard analysis for SMP characterization and identification, the comparison of results from different studies is also difficult. Most of the studies focus on the general characterization, even though greater knowledge of their composition is necessary to understand their production mechanisms. Kunacheva and Stuckey (2014) summarized methods for analyzing SMPs as shown in Table 2.1

Table 2.1: analytical procedures and investigated parameters

Parameter	Methods of analysis
MW distribution	Size Exclusion Chromatography (SEC); Gel-filtration chromatography (GFC); Gel-permeation Chromatography; Ultrafiltration (UF).
Identification of LMW compounds	Gas Chromatography – Mass Spectrometry (GC-MS) with matching libraries.
Hydrolysis of HMW compounds	Acidic hydrolysis; Microwave radiation induced hydrolysis; Alkaline hydrolysis; Enzymatic hydrolysis.
Protein content	Lowry method; Excitation-emission matrix spectroscopy (EEM); Resonance light scattering; Ion-exchange Chromatography.
Total carbohydrates	Titration; Gravimetric methods; Colorimetric methods; Gas Chromatography (GC); High Performance Liquid Chromatography (HPLC).
Aromaticity	UV-Vis Spectrometry.
Functional Groups	Nuclear Magnetic Resonance (NMR); Raman spectrophotometry; Fourier Transformed Infrared Spectrometry.
Biodegradability	BOD test (aerobic system); Biochemical methane potential (anaerobic system).

2.2.2 SMP treatments

SMP production can be lowered by the optimization of the biological process. Nevertheless, it is inevitable that some SMPs remain in the effluent from a biological treatment process. MW distribution, biodegradability and SUVA characterization are particularly useful for determining the efficiency and suitability of the posttreatment removal techniques.

Many researchers have investigated advanced techniques for SMP removal, such as activated carbon, membrane filtration techniques, synthetic resin absorption, ozonation, chemical precipitation and breakpoint chlorination. SMP can be precursor for trihalomethanes (THM), therefore chlorination may induce the formation of carcinogenic by-products (Namkung and Rittmann 1988). Granular activated carbon (GAC) appears to be the most effective method, with differences in performance depending on the MW (Barker and Stuckey 1999).

2.3 BACTERIOPHAGES

Bacteriophages, also named phages, are viruses that infect bacteria causing their lysis. Viruses are obligate intracellular parasites and can replicate only inside a living host cell, on which they rely for protein synthesis, energy and metabolic intermediates production. They have a nucleic acid genome and an extracellular form, named virion, which allows the movement from one host cell to another. The virion has a protection function of the viral genome when outside the host cell and it presents on its surface important protein for the attachment to the host cell. The infection process occurs when the genome of the virus has entered the host cell. Inside the host, the virus can express two different types of infection. In a lytic infection, the host metabolism is directed to support virus replication and virion formation. The infection continues until the host cell is destroyed and new virions are released. Alternatively, some viruses can undergo a lysogenic infection in which the host cell is not destroyed but is genetically altered because the viral genome becomes part of the host genome (Madigan, Martinko et al. 2014).

2.3.1 Relevance in wastewater treatment systems

The bacteriophage predation has raised interest in their potential application in wastewater treatment, since bacteriophages appear to be active components of activated sludge community (Hantula, Kurki et al. 1991). Epifluorescence microscopy has been applied to estimate the total viral counts in activated sludge reactors and many investigations reported a viral concentration of 10^8 to 10^9 virus like particles (VLP)/ml, resulting to be the most numerous entities (Shapiro and Kushmaro 2011). Even though there is little evidence of their activity directly altering activated sludge performance, the application phages in wastewater treatment may provide long term and cost effective techniques for improving effluent and sludge emissions into the environment (Withey, Cartmell et al. 2005). The bactericidal action of phages is generally accepted to enhance microbial diversification, which promotes resilience to perturbations of the community of activated sludge and therefore stability of performance (Shapiro and Kushmaro

2011). The microbial diversity control may be not the only the only potential of phage mediated bacterial mortality. Phages could potentially be used in biological sludge stabilization to regulate the abundance of specific pathogenic bacteria; improving dewaterability and digestibility of waste activated sludge; the control of filamentous bacteria; and reducing the competition between nuisance bacteria and functionally relevant bacterial strains (Withey, Cartmell et al. 2005). The current understanding of bacteriophages ecology is limited and it needs to be better investigated before starting phage manipulation in activated sludge systems (Shapiro and Kushmaro 2011). It is also possible that phage activity has an effect on the SMP concentration and composition in activated cell. A microbial cell lysed because of a phage infection will release a range of SMP into the surrounding water.

2.3.2 Detecting and counting bacteriophages: the plaque assay

In order for bacteriophages to replicate, host cells need to be cultivated either in liquid form or as lawns over agar plates (Madigan, Martinko et al. 2014). Plaque assays are the most spread technique for phage detection. With this technique, dilutions of the phage preparations are mixed with a permissive host bacterium and they are disseminated onto a semisolid medium, typically soft agar. During the incubation, the bacterial isolate forms a turbid layer over the medium, and when the infection takes place clear spots result in a localized zone, called plaques. This translucent area is determined by the cell lysis and it expands until it is visible to the naked eye. In order to expand, a sufficient yield of phage progeny needs to form from each bacterial cell (Kropinski, Mazzocco et al. 2009). By counting the number of plaques, it is possible to calculate the titer of the virus sample, expressed as plaque-forming units (PFU) per milliliter (Madigan, Martinko et al. 2014). This method permits the isolation of bacteriophages, the enumeration and also their characterization by plaque morphology. Plaques can be different in size, present an halo and show either a clear or a turbid lysis (Kropinski, Mazzocco et al. 2009).

2.4 RYA WWTP

Rya wastewater treatment plant (WWTP) was built in 1972 and purifies wastewater for an equivalent population of 730000 inhabitants and an average of 4000 liters of wastewater per second pumped to the plant. It serves the municipalities of Ale, Göteborg, Härryda, Kungälv, Lerum, Mölndal and Partille (Avfall Sverige 2013). The WWTP is part of the regional sewage works of Göteborg region, run by Gryaab AB, which is jointly owned by those municipalities. At the plant, the residual sludge product from the wastewater is treated, biogas is produced and converted to green vehicle fuel. The treated water is discharged into the river Göta älv in the proximity of the sea and the dewatered sludge is used for landscaping (Gryaab AB 2011).

Pollutants in wastewaters consists of a complex mixture of organic and inorganic contaminants (Ramalho 2012). The purpose of a WWTP is to reduce the discharge of those contaminants to the receiving environment by the mean of particle separation and biological processes. The treatment chain can be divided in three broad areas: mechanical, biological and chemical (Gryaab AB 2011). Table 2.2 contains a brief description of the treatment chain present in Rya WWTP.

Table 2.2: Rya WWTP treatment chain

Operation	Process description
Mechanical process	
Coarse bar screen, Sand trap, Fine bar screen, Primary sedimentation	Those steps constitute the primary treatment in the treatment plant and are designed to remove coarse material, gravel, sand and suspended solids from the raw sewage.
Secondary sedimentation	The secondary sedimentation tank is located after the activated sludge tanks and it is designed to separate the sludge from the water. The biological sludge is formed by bacteria and precipitated phosphate and it is partly recirculated to the activated sludge tanks, partly removed and treated
Disc filters	The secondary effluent is filtered through disc filters before being discharged to the river. The filter consists of multiple cloths vertically installed on rotating drums. Gryaab's disc filters have screen cloths with 15 micrometres holes.
Chemical process	
Iron sulphate dosing	Iron salts promote the precipitation of the soluble phosphorus present in the wastewater insoluble compound. Phosphorous is therefore precipitated as phosphate, removed and treated in the biological sludge.
Polyaluminium chloride	During heavy rain or snow melting, the wastewater that overcome the treating capacity of the plant is collected in several preliminary settling basins where the phosphate is removed by adding polyaluminium chloride to the water. Normally flows up to 10 m^3/s receive full biological and chemical treatment
Biological process	
Anoxic activated sludge tank	The first chambers of the activated sludge tanks are not aerated in order to favor the denitrification step in nitrogen removal process, which requires anoxic conditions to

	occur, since bacteria are forced to use nitrate instead of oxygen for respiration.
Aerobic activated sludge tank	In the aerobic tanks, the air is pumped in order to promote organic carbon removal.
Trickling filters	Blocks of corrugated plastic sheets support the growth of a bacteria in a biofilm. In this step, ammonia is converted to nitrite and nitrate in a process called nitrification.
Moving Bed Biofilm Reactor (MBBR)	The plant needed to be supplemented with additional denitrification, therefore in 1997 an additional step was built to enhance denitrification. The bacteria grow on a moving plastic media, which allow the microorganisms responsible for nitrogen removal to be active over a longer period of time compared to activated sludge condition. An external carbon source is required and it is dosed as methanol.

3 METHODOLOGY

The experiments were carried in the Environmental Chemistry laboratory at the department of Civil and Environmental Engineering, Chalmers University of Technology. The activated sludge was collected in January 2016 from the Rya wastewater treatment plant located in the city of Gothenburg. The AS was stored in a refrigerator at a temperature of 4°C.

3.1 EXPERIMENTAL SETUP

3.1.1 Investigation of the activated sludge in batch culture systems

The tests were conducted using two different sets of reactors that were operated under the same conditions, except for the concentration of the carbon sources. Before starting the investigation of the batch reactors, the activated sludge was acclimatized to the new substrate and environmental conditions. A schematic of the procedure is shown in Figure 3.1.

Acclimatization step

The activated sludge was inoculated into four flasks that differs for the carbon source utilized (Table 3.1: composition of the four different media). The bacterial communities grew under aerobic environment. The liquid was kept under continuous mixing by a magnetic stirrer. The carbon source was refilled every ten to fifteen days to keep the population under growing conditions for a period of 5 weeks before starting to run the batch reactors. The purpose of this procedure was to obtain different cultures growing on specific carbon sources. Since each growth medium only contained one specific carbon source that could be measured using high-performance liquid chromatography (HPLC), soluble microbial products and the carbon source could be differentiated in the reactors.

First set of batch reactors

At the starting point of the experiments, 5 mL of each community from the Erlenmeyer flasks were inoculated into one-liter bottles, respectively for each media, following the concentrations listed Table 3.1. Oxygen was provided by means of aquarium pumps and porous stones. A magnetic stirrer kept a continuous mixing state, which together with the ventilation prevented the sedimentation of the forming flocs. The aeration provided by the pumps caused some loss of liquid, therefore the lost water was refilled after the sampling up to one liter every second day. The reactors were covered by aluminum paper to avoid solar radiation and growth of algae in the systems.

Second set of batch reactors

A consecutive set of experiments was performed on another two batch reactors, the first containing acetic acid and the second butyric acid as carbon sources, presenting the same mineral solution but with a concentration of sodium acetate and sodium butyrate

three times higher than the previous reactors. The inocula used in the second reactors were extracted from the respective first set of batch reactors.

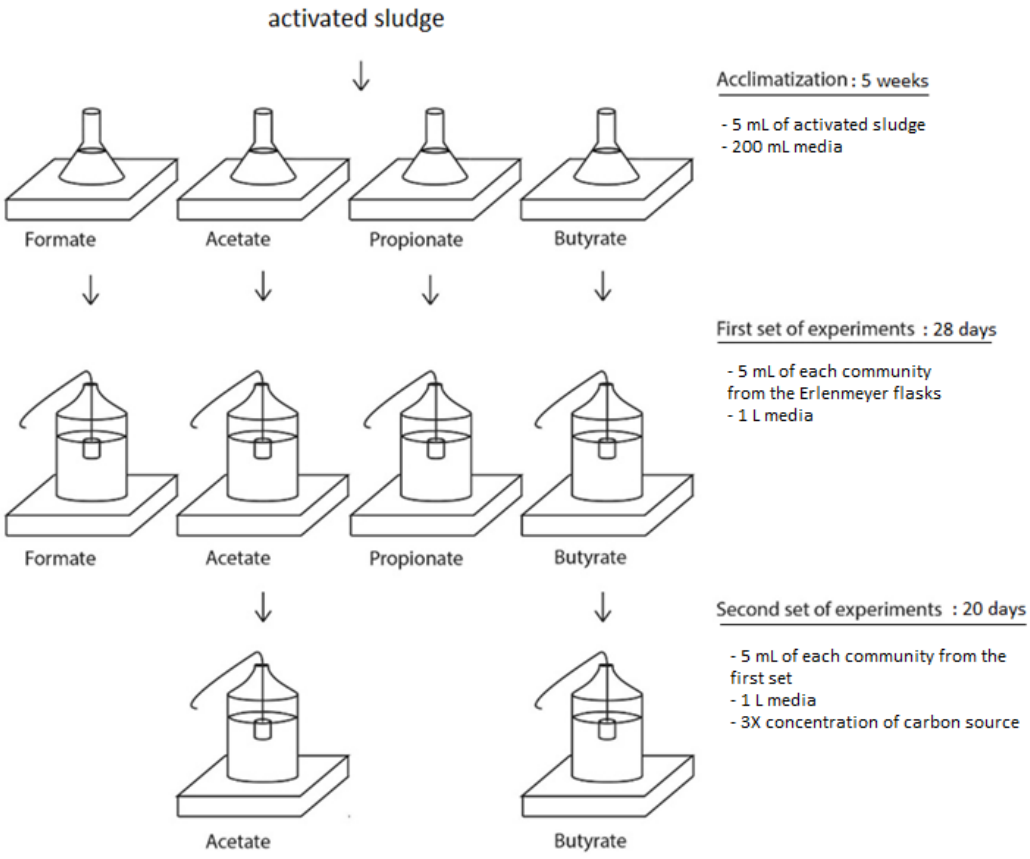

Figure 3.1: schematic of the experimental setup

3.1.2 Culture media

In order to grow, bacterial cells need a set of essential nutrients, which include: a carbon source for cell material production; an energy source for carrying out the biochemical reactions; a nitrogen source, for protein and nucleic acid synthesis; sulphur for amino acid formation; macronutrients such as phosphorus, potassium, magnesium and calcium for building cell components and enzymatic functions (Seviour, Blackall et al. 1999). Those nutritional requirements were satisfied in the culture media used for the bacterial growth. The compounds were diluted in deionized water in concentrations indicated in Table 3.1 and Table 3.2. As previously mentioned, the four batch reactors differed just for the carbon source utilized. The compounds added for organic carbon sources are salts of short-chain fatty acids, also referred to as volatile fatty acids (VFAs).

Table 3.1: composition of the four different media

Compounds	Medium 1	Medium 2	Medium 3	Medium 4
Organic carbon source				
Sodium formate $CHNaO_2$	1133 mg/L	-	-	-
Sodium acetate $C_2H_3NaO_2$	-	683 mg/L	-	-
Sodium propionate $C_3H_5NaO_2$	-	-	534 mg/L	-
Sodium butyrate $C_4H_7NaO_2$	-	-	-	458 mg/L
Mineral solution				
$NaHCO_3$	1000 mg/L			
$NaNO_3$	700 mg/L			
NaH_2PO_4	50 mg/L			
$MgSO_4 \cdot 7H_2O$	200 mg/L			
$CaCl_2 \cdot 2H_2O$	200 mg/L			
KCl	200 mg/L			
Trace elements	1 mL/L			
Se/W	1 mL/L			

Table 3.2: Composition of trace elements and Se/W solutions

Trace elements	
$FeCl_2 \cdot 4H_2O$	2 g/L
H_3BO_3	0.05 g/L
$ZnCl_2$	0.05 g/L
$CuCl_2$	0.03 g/L
$MnCl_2 \cdot 4H_2O$	0.5 g/L
$(NH_4)Mo_7O_2 \cdot 4H_2O$	0.05 g/L
$AlCl_3$	0.05 g/L
$CoCl_2 \cdot 6H_2O$	0.05 g/L
$NiCl_2$	0.05 g/L
EDTA	0.5 g/L
HCl conc.	1 g/L
Se/W	
Na_2SeO_3	0.1 mmol/L
$Na_2WO_4 \cdot 2H_2O$	0.1 mmol/L

3.2 MODELLING OF THE BATCH FERMENTER

Equation 1 was used to describe the change in biomass concentration in the batch reactors

$$\frac{dX}{dt} = \mu X = -Y \frac{dS}{dt} - k_d X \quad (1)$$

where X is the biomass concentration (mg MLVSS/L), S is the limiting substrate concentration (mg VFA/L), t is time (d), μ is the growth rate (d^{-1}), Y is the yield coefficient (mg MLVSS/mg VFA), and k_d is the decay coefficient (d^{-1}). It was assumed that the substrate was converted only into biomass and the maintenance rate was neglected. The decay coefficient was determined from the endogenous respiration phase of the batch experiments. The yield coefficient was determined from initial growth phase.

3.3 ANALYSIS

The samples were taken from the four batch reactors every second day when possible. Sample tubes were stored in the freezer at a temperature of -20ºC until analysis. The analyses were performed according to Table 3.3.

Table 3.3: analytical procedures and investigated parameters

Parameter	Method of analysis	Preparation of the sample
Optical Density (OD)	Spectrophotometer UV-1800 (Shimadzu)	Measurement performed at a wavelength of 600 nm using 1 cm cell.
Absorbance spectrum, SUVA, Slope Ratio	Spectrophotometer UV-1800 (Shimadzu)	Filtration of the sample through 0.45 µm membrane before measurement using 1 cm cell.
Dissolved Organic Carbon (DOC)	TOC-V_{CPH} (Shimadzu)	Filtration of the sample through 0.45 µm membrane to remove particulate OC, before diluting with Milli-Q water.
Mixed Liquor Suspended Solids (MLSS)	TSS standard method procedure (Rice, Bridgewater et al. 2012)	Filtration of the sample through glass paper fiber. Loss on drying in the oven for 90 minutes at a temperature of 105 °C.
Mixed Liquor Volatile Suspended Solids (MLVSS)	VSS standard method procedure (Rice, Bridgewater et al. 2012)	Loss on ignition for 15 minutes at a temperature of 550 °C.
Volatile Fatty Acids (VFAs)	High Performance Liquid Chromatography (HPLC)	Filtration of the sample through 0.45 µm membrane before diluting with Milli-Q water. Eluent: 5mM H_2SO_4.

Molecular Weight (MW) distribution of organic matter	High Performance Size Exclusion Chromatography (HPSEC) with online UV spectrophotometer	Filtration of the sample through 0.45 μm membrane without dilution. The UV detector monitored UV absorbance at the wavelength of 270 nm and 350 nm. Eluent: 100 mM NaCl, mM KH_2PO_4, mM K_2HPO_4.

MLSS and MLVSS were calculated according to the following equations:

$$MLSS = \frac{W_{post} - W_{pre}}{V} \quad (4)$$

$$MLVSS = \frac{W_{post} - W_{combustion}}{V} \quad (5)$$

where W_{post} is the weight of the filter after the loss on drying in the oven, W_{pre} is the weight of the clean glass paper fiber, $W_{combustion}$ is the weight of the filter after the loss on ignition in the furnace.

The total SMP were determined from the differences as follows:

$$Total\ SMP\ (TOC) = Total\ DOC - VFAs \quad (6)$$

The spectral slope S (nm^{-1}) was derived from the absorbance spectra by fitting the data to the equation (Coble 2007):

$$a_{\lambda} = a_{\lambda_{ref}} e^{-S\,(\lambda - \lambda_{ref})} \quad (7)$$

where a_{λ} is the absorption coefficient, λ is the wavelength (nm), λ_{ref} is the reference wavelength (nm). The absorption coefficient was calculated as (Coble 2007):

$$a_{\lambda} = 2.303\ A/l \quad (8)$$

where A is the absorbance, l is the path length (m) and 2.303 converts between log10 and natural log.

The slope ratios were calculated as the ratio of two distinct spectral slope regions (Helms, Stubbins et al. 2008):

$$S_R = \frac{slope\ of\ 275-295\ nm\ region}{slope\ of\ 350-400\ nm\ region} \quad (9)$$

The Specific UV Absorbance (SUVA) was calculated from the ratio of a_{254} (m^{-1}) to DOC concentration (mg/L) (Weishaar, Aiken et al. 2003).

The choice of the wavelengths used for the MW distribution investigation was based on the analysis of the absorbance spectrum of the samples. The values of 270 and 350 nm corresponded to two of the peaks registered in the spectrum curve. The results obtained from the HPSEC were compared with the standards acquired using polyethylene glycol listed in Table 3.4.

Table 3.4: HPSEC standards using polyethylene glycol.

MW (Da)	21300	16100	7830	4040	1480	1010	610	420	194	106
UV (RT min)	31.8	32.95	35.6	38.35	42	43.2	44.3	45.1	46.2	46.8

3.4 ISOLATION OF BACTERIA

Samples from the batch reactors were serially diluted in a 7-fold dilution bank. The serial dilution was performed by transferring 1 ml of the environmental sample in 9 ml MQ water tube and repeating the procedure according to the desired dilution. A volume of 0.1 ml of bacterial suspension from different dilution tube was spread over the plates with a cell glass spreader. Sterile saline solution NaCl (0.85 g/100 mL of MQ) was used for the dilution of the samples. The dilution was implemented in order to obtain separated colonies on the plate. After 7 days of incubation at room temperature, isolated colonies from the plates with 10^{-4} and 10^{-5} dilutions were picked and purified by streaking on the same medium used for primary isolation. After spreading, the bacteria that grew well separated from the others were picked and the streaking procedure was repeated to ensure the isolation. In order to maintain an aseptic environment, all the steps were performed in the proximity of a Bunsen burner.

The culture media in the plates reflected the composition of batch reactor solutions and was solidified with 1.5 % agar after sterilization with autoclave. All the plates were incubated inverted at room temperature.

For bacteria and bacteriophages isolation, a more general media was used parallel to the four listed in Table 3.1, promoting the growth of faster population of bacteria. The media contained the same mineral solution as the others and in addition the compounds listed in Table 3.5.

Table 3.5: general media composition

Compound	Concentration
Sodium Acetate	683 mg/L
Glucose	600 mg/L
Yeast extract	200 mg/L
NH_4Cl	382 mg/L

3.5 ISOLATION OF BACTERIOPHAGES

The double layer plaque assay was the protocol used to isolated bacteriophages. Phages were inoculated from the supernatant of the activated sludge collected from Rya WWTP. The supernatant was filtrated with 0.22 μm membrane to exclude bacteria from the activated sludge sample and diluted in 10-fold dilutions with the same media used for bacterial isolation. For each dilutions, 100 μL of the phage preparation was mixed with 100 μL ml of overnight growing cultures of host bacteria and dispersed into 3 ml soft agar media (0.4% agar). The tubes with soft agar media were kept in a water bath at 45°C in order to avoid the solidification of the media and maintain a harmless temperature for the bacteria. The tubes were then mixed and poured on the top of a hard agar Petri dish. Control plates with soft agar and bacterial isolates were prepared without phage preparation. The soft agar layer prevented the virus from spreading from the host cell to other surrounding uninfected cells. After the hardening of the soft agar, the plates were inverted and incubated at room temperature. Plates were checked daily for plaque formation.

Once observed, the plaques containing viruses were supposed to be picked and stored in sterile media. The viruses thus isolated were intended to be tested on a different host, in order to study the infectivity range over different bacterial isolates.

3.6 ENUMERATION OF BACTERIOPHAGES

Wastewater samples from Rya WWTP were taken in three different positions of the chain process (influent, aerated tank with activated sludge, effluent, as shown in Figure 3.2), then filtered through 0.22 μm membrane and sent to Izon Science for enumeration of viruses in terms of virus like particles (VLP) per liter. The measurement methodology, known as Tunable Resistive Pulse Sensing (TRPS), exploits dynamically resizable nanopores for real-time particle detection, quantitation and characterization (IZONscience 2015). The pore size NP150, which is suitable for VLP between 80 and 200 nm, was used.

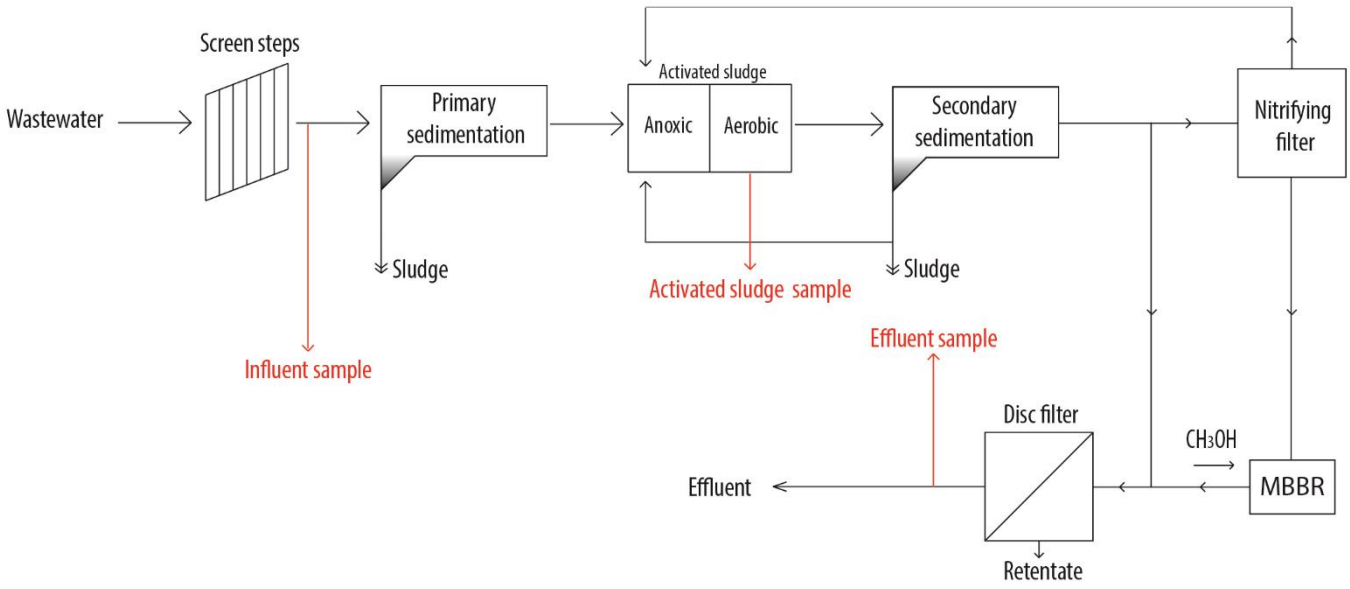

Figure 3.2: scheme of the WWTP and points of sampling

In case of plaque formation, the phage concentration in terms of VLP/mL could have been compared with the titre of the original phage preparation as plaque forming unit (PFU), calculated as:

$$Number\ of\ plaques\ \times 10 \times\ Reciprocal\ of\ dilution\ = \frac{PFU}{ml} \quad (10)$$

3.7 MICROSCOPY ANALYSIS

Light microscopy analysis was performed over the reactor samples and the isolation plates in order to investigate the morphology of flocs and the community of isolates. Two different light microscopes were utilized, both present in the Environmental Chemistry laboratory. Samples were observed with different magnification and illumination techniques in order to generate a better contrast of the image. Bright field illumination was used for the highest magnification, while polarized illumination was used for images with 60X magnification.

4 RESULTS AND DISCUSSION

4.1 INVESTIGATION OVER THE BATCH REACTORS

4.1.1 Biomass growth

Based on the growth media, different population of bacteria arose and established in the reactors. Considering the carbon source and the supply of oxygen to satisfy the biochemical oxygen demand in the process, it is likely that the dominant populations were organoheterotrophic bacteria, oxidizing the organic carbon to CO_2. Based on the assumption that the organic carbon was degraded through an aerobic respiration process, different free energy values ($\Delta G°$) are obtained depending on the nature of the substrate. Table 4.1 shows the calculated free energy values for one mole of each salt of VFAs. The higher $\Delta G°$, the higher is the bacterial yield and thus biomass level (Kuo, Sneve et al. 1996).

Table 4.1: Gibbs free energy values for the organic carbon compounds

Compound	Structural formula	Biological decomposition of one mole of substrate under aerobic conditions	Gibbs free energy $\Delta G°_{298}$ (kJ/ C)
Formate	$HCOO^-$	$HCOO^- + 0.5O_2 \rightarrow HCO_3^-$	-236.06
Acetate	CH_3COO^-	$CH_3COO^- + 2O_2 \rightarrow HCO_3^- + CO_2 + H_2O$	-423.2
Propionate	$CH_3CH_2COO^-$	$CH_3CH_2COO^- + 3.5O_2 \rightarrow HCO_3^- + 2CO_2 + 2H_2O$	-494.77
Butyrate	$CH_3(CH_2)_2COO^-$	$CH_3(CH_2)_2COO^- + 5O_2 \rightarrow HCO_3^- + 3CO_2 + 3H_2O$	-532.32

As can be seen from the calculations, the higher is the number of carbon atoms in the compound, the higher is the $\Delta G°$ obtained. Butyrate, which has four carbon atoms, leads to the highest result, followed by propionate, acetate and formate, respectively. Therefore, it was expected that butyrate fed reactor led to a greater biomass level compared to the other three VFAs.

4.1.2 Cell mass concentration

The bacterial growth in a culture can be determined by measuring the cell number. The cell density was measured optically by a spectrophotometer, which determines the turbidity of a solution by measuring the amount of light that passed through a suspension of cells. The cells scatter the light beam passing through the cuvette and the scattering intensity or turbidity, also called optical density OD, is proportional to the bacterial density (Widdel 2007). The OD of a culture depends on the wavelength used;

as previously mentioned, a wavelength of 600 nm was chosen. Based on the results presented in the graphs below (Figure 4.1 and Figure 4.2), it was possible to estimate the different growth phases for the bacterial population in the batch culture.

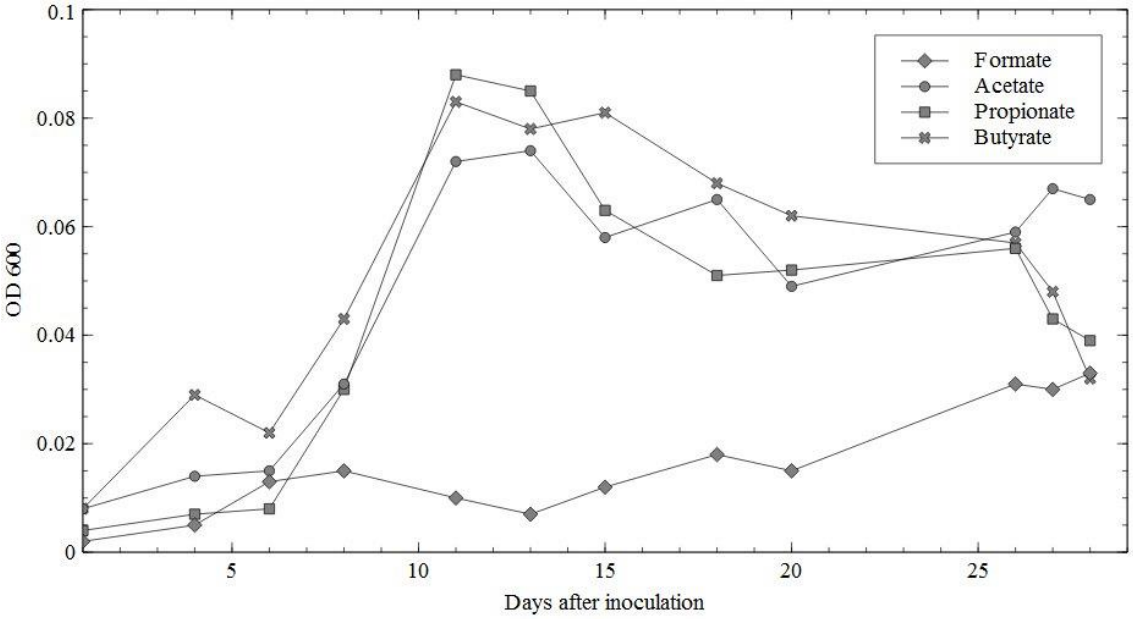

Figure 4.1: optical density curves for the first set of batch reactors

The shape of the graph in Figure 4.1 shows similar biomass growth curves for the reactors fed with butyrate, propionate and acetate, while in case of formate the pattern is different. In this reactor, as expected from the free energy results, the growth appeared to be slower and it led to a lower biomass level, reaching a maximum of OD=0.033 at the end of the experiments. By the end of the test, the biomass grown on formate still presented a growing trend as opposed to propionate and butyrate reactors, which showed a decline. The reactor fed with acetate exhibited a higher development of biomass than formate, reaching a peak of OD=0.074. After this day, the cell density showed a decline, followed by a stationary phase, even though the organic carbon source provided was totally consumed by the 11^{th} day as shown by the results of the HPLC (Figure 4.6). A possible explanation to those results may lie in the fact that another population of bacteria with different metabolic needs overcame the previous dominant one responsible for acetate depletion. The propionate reactor presented the greatest cell density values equal to an OD of 0.088, slightly higher than the peak reached by butyrate (OD=0.083). On the other hand, butyrate presented a longer stationary phase from day 11^{th} to day 15^{th} compared to propionate, and it showed a significantly faster and higher development of biomass level in the first 10 days of incubation, leading to a quicker depletion of the carbon source.

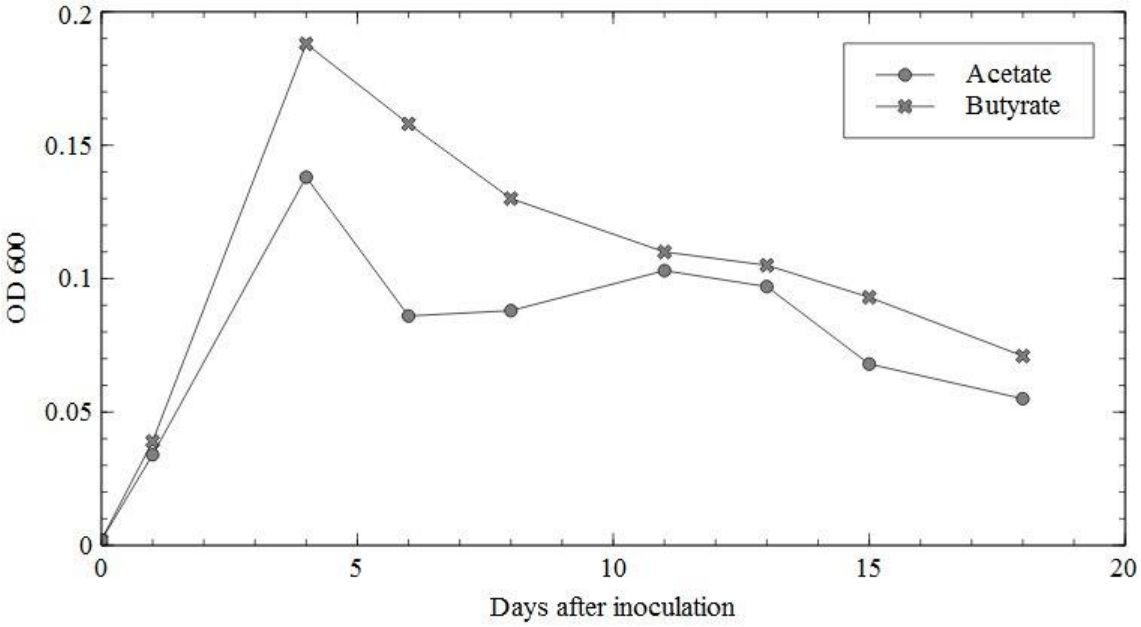

Figure 4.2: optical density curves for the second set of batch reactors

In the second set of experiments, butyrate and acetate concentration were triplicated to feed the activated sludge. As it can be seen in Figure 4.2, a higher concentration determined a greater and a faster biomass development. Butyrate reached a peak of OD=0.188 and acetate culminated to 0.138 four days after the inoculations, while in the first set of experiments, the maximum cell density was reached after 11 days. The faster development of biomass can be also pointed at the different age of inoculum used. In the second set of reactors, the communities of inoculated bacteria were longer adapted to VFAs degradation compared to the inocula used in the first set of reactors. Similarly to the previous case, the decline of biomass concentration in the butyrate reactor was sharper than in the acetate reactor.

Overall, both set of experiments show that OD peaks were reached in correspondence of VFA complete degradation (Figure 4.6 and Figure 4.7).

Biomass concentration was investigated also in terms of MLVSS. The correlations between OD and this parameter in the reactors are shown in Figure 4.3 and Figure 4.4. There is a linear relation between the parameters within the range investigated in this study for all the reactors. This correlation was not observed for the reactor fed with formate. The formate OD results showed an increasing pattern, while MLVSS analysis reported decreasing biomass concentration during the experiments, denoting a possible mistake in the analysis of biomass concentration.

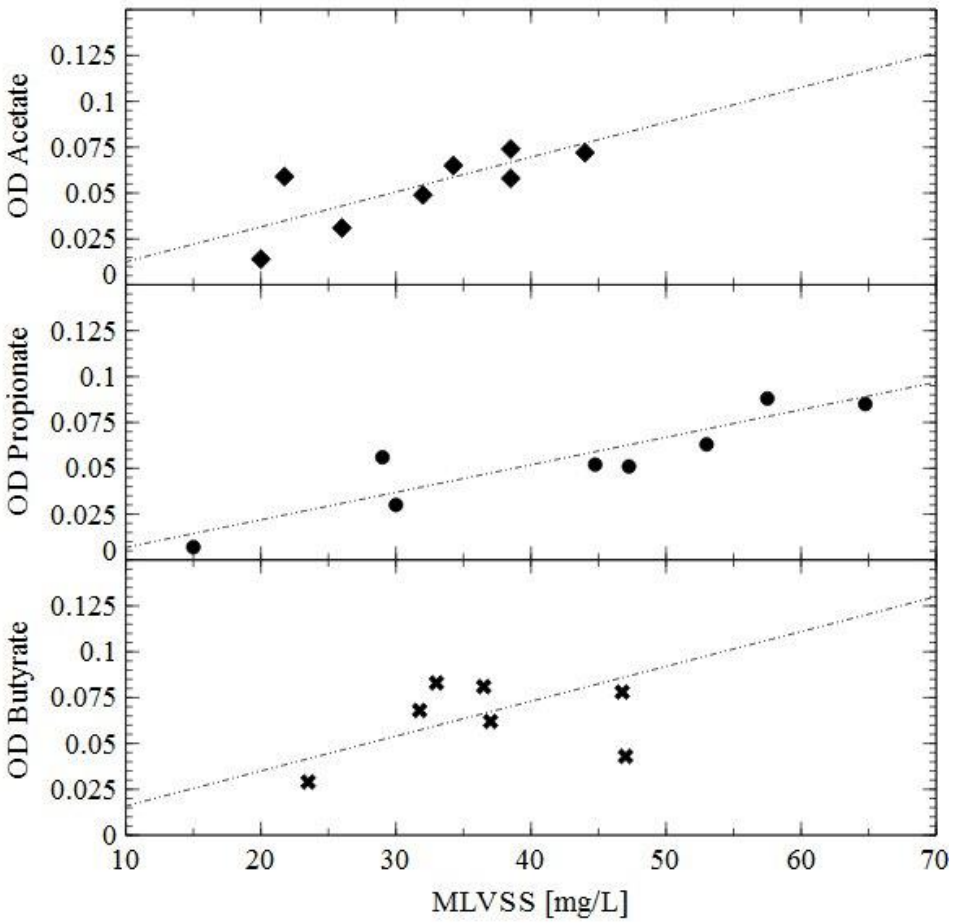

Figure 4.3: OD and MLVSS correlation in the first batch reactors

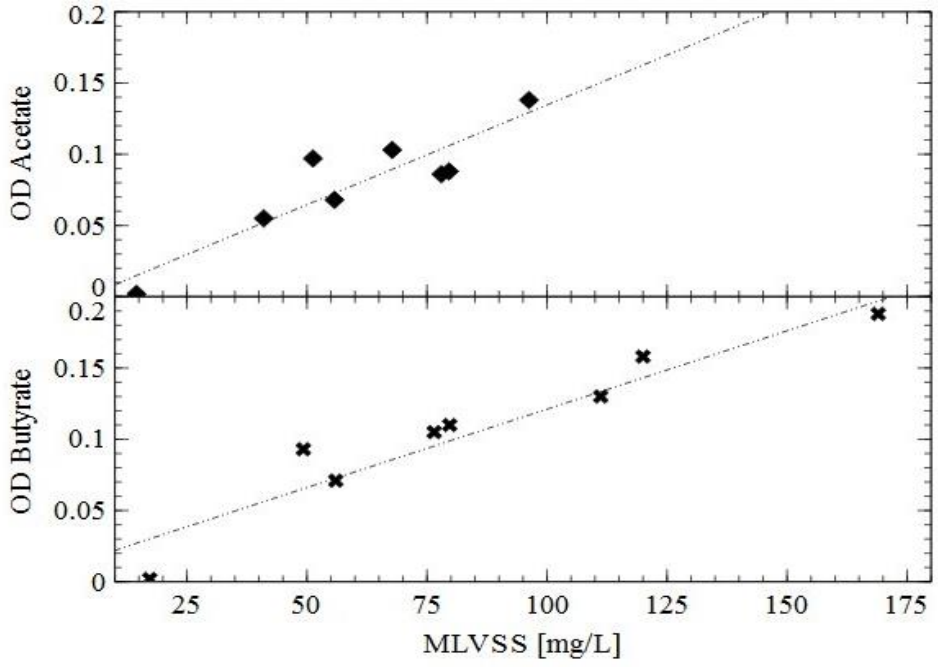

Figure 4.4: OD and MLVSS correlation in the second batch reactors

4.1.3 Stoichiometric and kinetic coefficients

From the microbial growth values and substrate consumption, it was possible to extrapolate parameters such as the yield coefficient, the growth rate and the decay coefficient. The yield coefficient is defined as the mass of activated sludge produced per unit of substrate removed. K_d is the endogenous decay rate during endogenous respiration per unit of time. The specific growth rate, μ, is the rate of growth per unit of time. For the experimental data and graphs with fitted lines, check Appendix.

Table 4.2: yield coefficient, specific growth and death rate of the reactors

Compound	Y [mg VSS/mg VFA-C]	μ [day^{-1}]	k_d [day^{-1}]
First set of reactors			
Acetate	0.22	0.11	0.045
Propionate	0.35	0.25	0.056
Butyrate	0.29	0.19	0.034
Second set of reactors			
Acetate	0.17	0.62	0.058
Butyrate	0.29	0.78	0.090

There is a linear relation between the microbial growth yield and the free energy of the catabolic reaction (Roden and Jin 2011). This is true, except for the propionate fed reactor, which presented the highest yield coefficient. Nevertheless, the yield depends not only on the nature of the substrate but also on the physiological properties of the organism and the energy required for the cell synthesis (Roden and Jin 2011). Since the more efficiently a substrate is utilized for cell growth the higher Y, it is possible to conclude that the biomass grown on propionate was the most effective in converting the substrate into biomass, requiring less energy for the cell synthesis compared to the other. The reactors fed with acetate showed the lowest growth rate for each set of experiments, meaning that the larger proportion of the substrate consumption was used for maintenance. The increase in the initial substrate concentration in the second set of experiments determined a rise of the specific growth and decay rate values.

4.1.4 Pigment production

The reactor fed with butyrate showed after one week of incubation a yellow color (Figure 4.5). Under specific growth condition, bacterial cells accumulate pigments (Kirti, Amita et al. 2014). The bright yellow color disappeared towards the end of the experiments, when the butyrate was completely consumed by the bacteria.

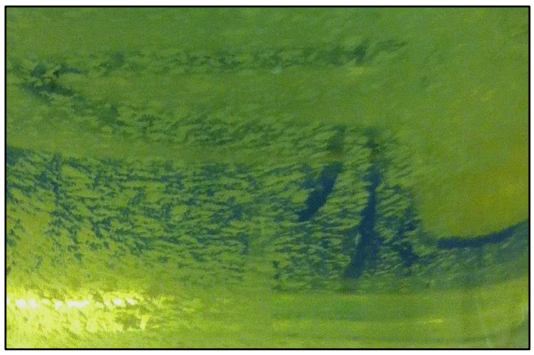

Figure 4.5: picture of the yellow activated sludge fed with butyrate

In the second batch, no yellow-pigmented bacteria were observed. This may be due to the fact that several factors affect microbial pigment production such as temperature of incubation, pH of the medium, the type of carbon and nitrogen source (Joshi, Attri et al. 2003). None of these features was modified except for the concentration of the carbon source, which was triplicated. An explanation to this result may lie in the different initial microbial population inoculated in the reactors. In the second reactor, the initial population was taken from the microorganisms of the first reactor. Another possible explanation may be a spontaneous mutation, which occurs due to occasional errors in the pairing of bases by DNA polymerase during DNA replication leading to the loss of enzyme in biosynthetic pathway responsible for pigment productions (Madigan, Martinko et al. 2014).

4.1.5 Substrate consumption and SMP production

The depletion of the different substrate and the production of SMP are shown in Figure 4.6 and Figure 4.7.

Regarding the first set, it was observed that VFA concentrations decreased rapidly from the fourth to the 11^{th} day after the inoculation in all the reactors except for the formate. Hence, the slower depletion of the first four days can be pointed to an acclimatization period. The slow depletion of carbon source in formate fed reactor justified the slow growth of the activated sludge observed in the OD results (Figure 4.1). The peaks in SMP concentration were registered in the growing phases of the activated sludge. The results, showed in Table 4.3, seem to reflect the free energy results. The higher the $\Delta G°$ obtained from the compound the higher is the SMP maximum concentration.

Table 4.3: SMP production peaks in the first set of batch reactors

Carbon Source	Formate	Acetate	Propionate	Butyrate
Maximum SMP concentration [mg C/L]	39.25	48.72	60.50	82.55
Days after inoculation	0	0	6	4

The samples of the first day of experiments were taken from two to three hours after the inoculation. The high SMP concentration measured for those samples may suggest that SMP production was a relatively fast process.

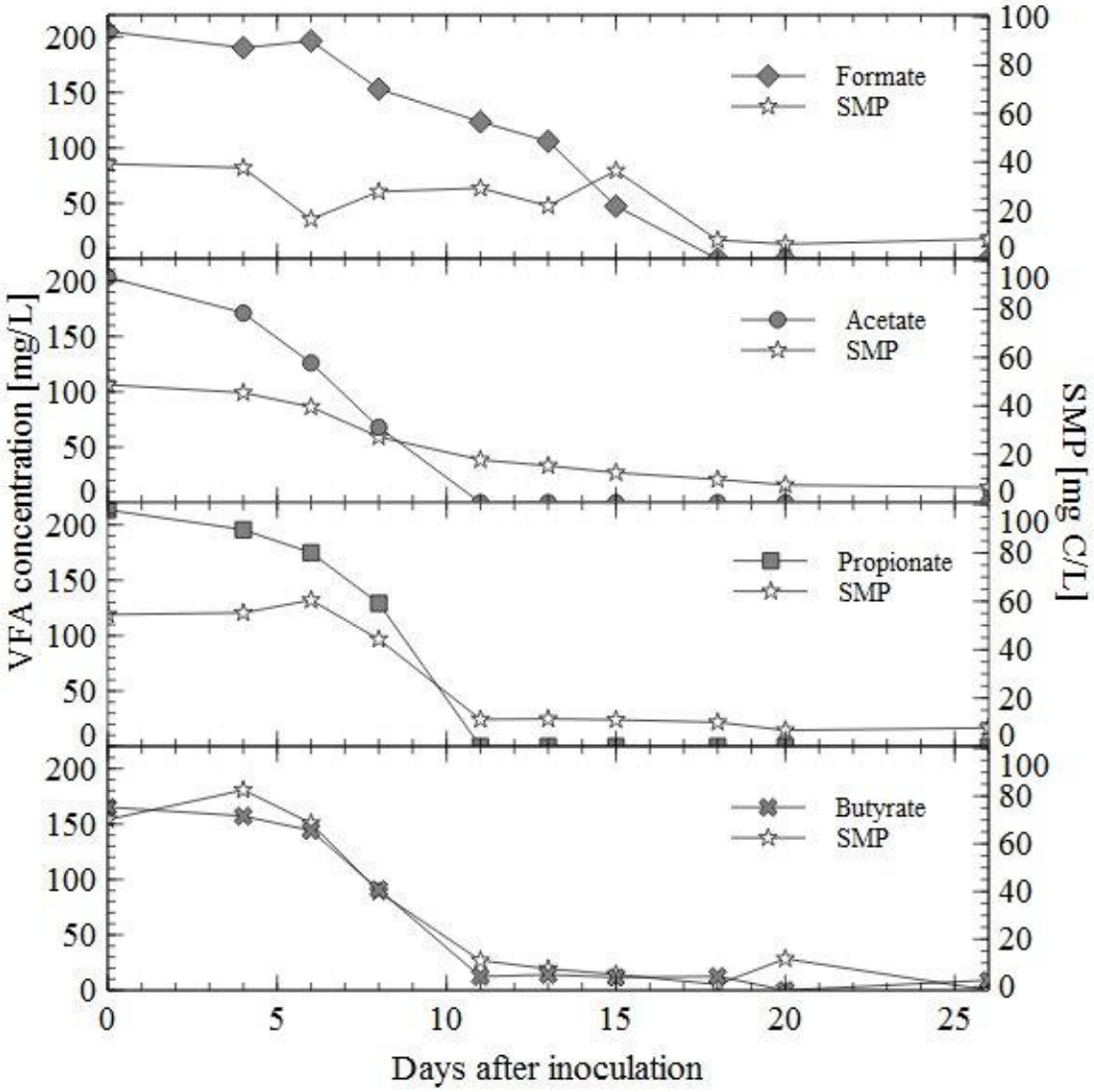

Figure 4.6: substrate consumption and SMP production in the first set of batch reactors

In the second set of batch reactors, a different substrate utilization rate was observed compared to the first set and VFAs were totally depleted in the first four days of inoculation. The production of SMP was significantly lower compared to the first set, even though the microbial community development and carbon utilization were higher and faster. According to Barker and Stuckey (2001), SMPs are formed in a rate proportional to biomass and substrate uptake, therefore greater concentration of SMPs were expected in the second set compared to the first one. The peak registered were 29.44 mg/L and 33.58 mg/L respectively for acetate and butyrate. These results may suggest that the longer a community is acclimatized to a carbon source the lower is SMP production.

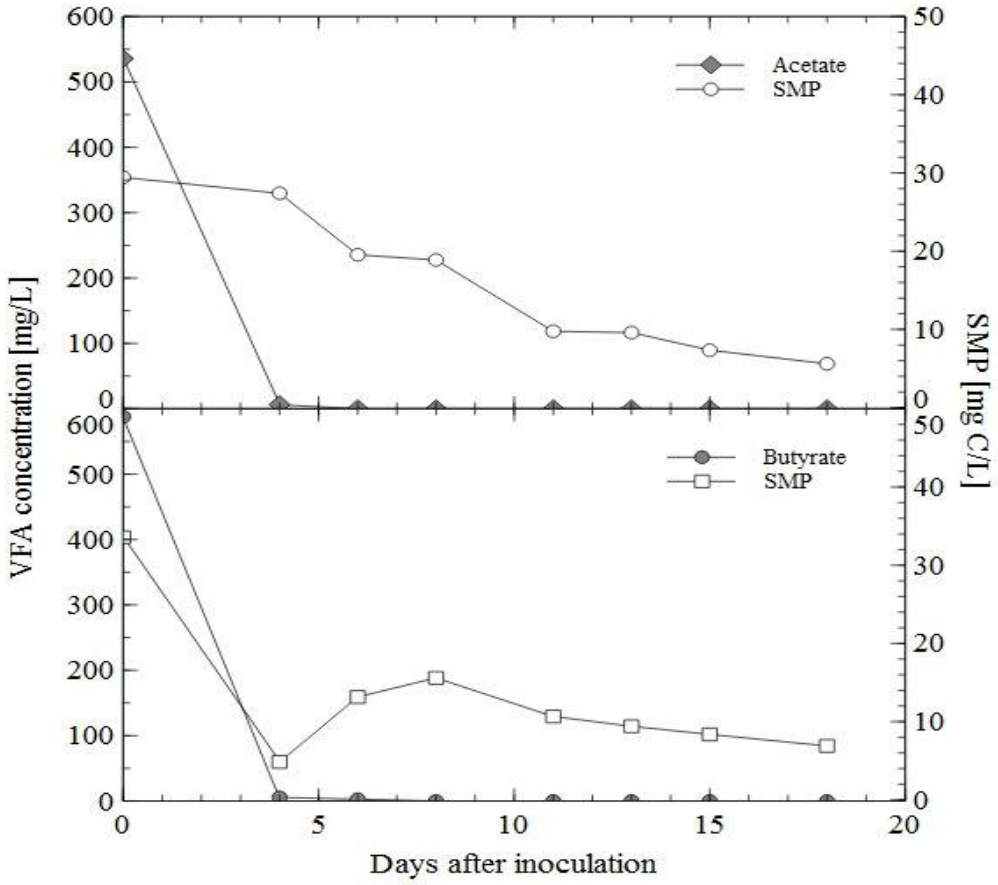

Figure 4.7: substrate consumption and SMP production in the second set of batch reactors

Figure 4.8 shows the normalized SMP accumulation in relation to the total amount of biomass present in the reactors (SMP/MLVSS). In all the systems, a decrease in accumulation was observed since the beginning of the experiments. In the formate fed reactor, the accumulation increased until the 16th day and dropped at the 18th day in correspondence of formate complete depletion. The increase in accumulation may be due to the slow biomass growth in this reactor. The highest accumulations occurred in the growth phases of the communities in abundant substrate condition. Hence, SMPs produced in this stage derived from an excessive metabolic activity and excretion of

organic matter related to substrate utilization. According to results of Jarusutthirak and Amy (2007), the SMPs accumulated in the first days of the experiments can be considered UAPs. MLVSS data were not performed at the inoculation of the first set of reactors; therefore, there are no normalized SMP values for this day.

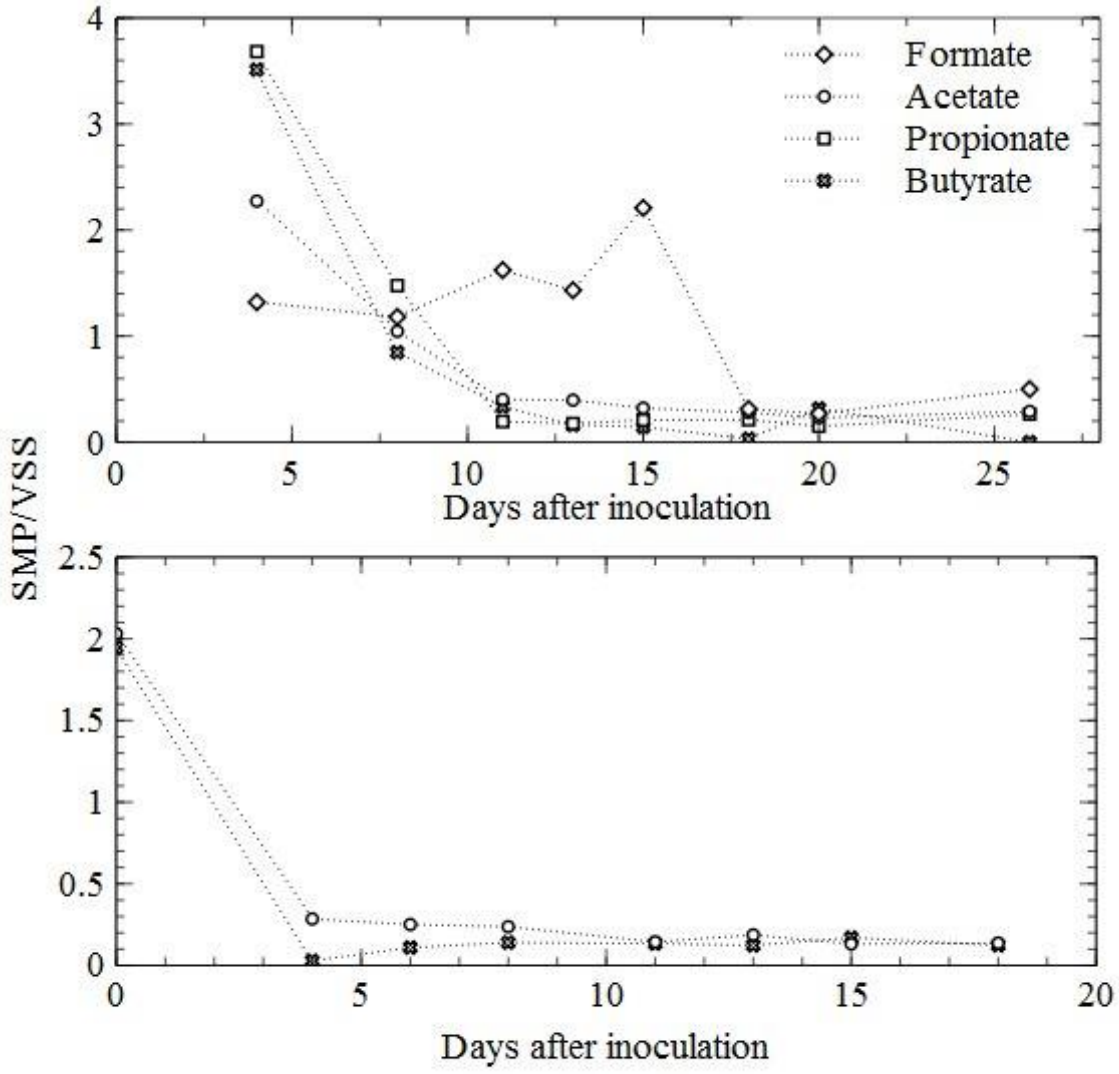

Figure 4.8: normalized SMP accumulation in relation to the total amount of biomass

4.1.6 Spectroscopic methods

For these measurements, just the acetate and butyrate samples from both sets of experiments were investigated.

Spectrum Curves

The spectra of the samples were examined in a wavelength range of 200-800 nm. The absorbance spectra of the sterile acetate and butyrate media were also analyzed and compared to the samples, in order to assess which compound of the media contributed the most to the absorbance and which wavelength range was relevant in the study. It

was established that sodium nitrate $NaNO_3$ was the responsible chemical for the high absorbance (Abs>3) in the range from 200 to 230 nm and for the smaller peaks registered around 300 nm (see Appendix).

From the comparison of the measured spectra, it was concluded the relevant wavelength range for this investigation varied from 250 to 800 nm. Within this range, the production and depletion of undefined organic matter were observed as variation of intensity of absorbance at different wavelengths. In every reactors, the spectrum curves registered at the beginning of the experiments (0 day) presented the same shape as the sterile media spectrum, but with higher intensity.

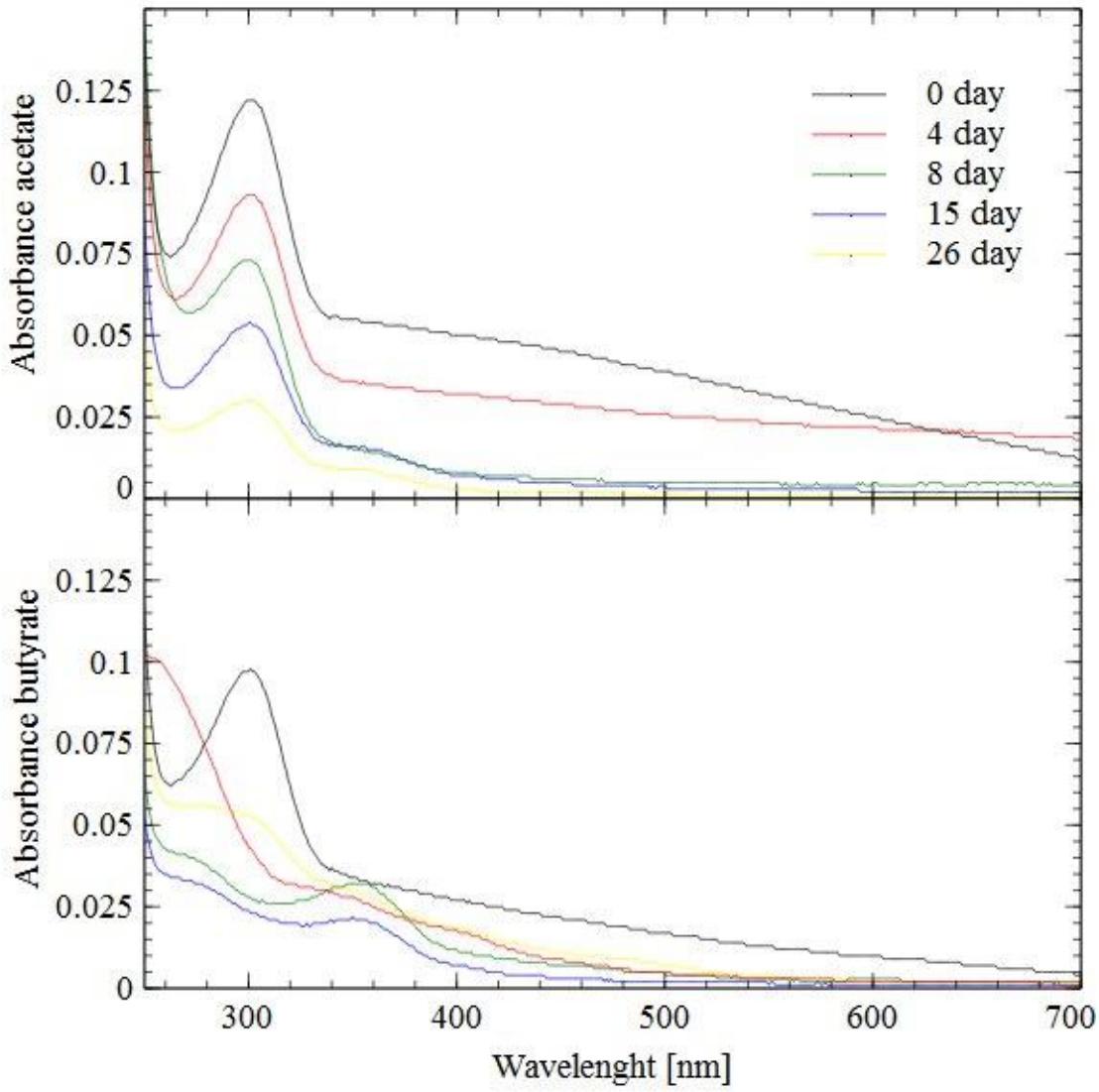

Figure 4.9: absorbance spectra of acetate and butyrate reactors of the first set

Regarding acetate fed reactor in the first set of experiments (Figure 4.9), it is possible to observe the decrease of the peak registered at 300 nm along the duration of the experiments. Hence, the decrement of the absorbance can be interpreted as the result of the biodegradation of DOC. The results obtained from butyrate fed reactor show a different pattern. Eight days after inoculation, another two peaks appeared on the curve around 260 and 400 nm, suggesting the possible formation of other kinds of SMPs with different molecular structure. Those peaks disappeared by the end of the experiment, reflecting biomass degradation.

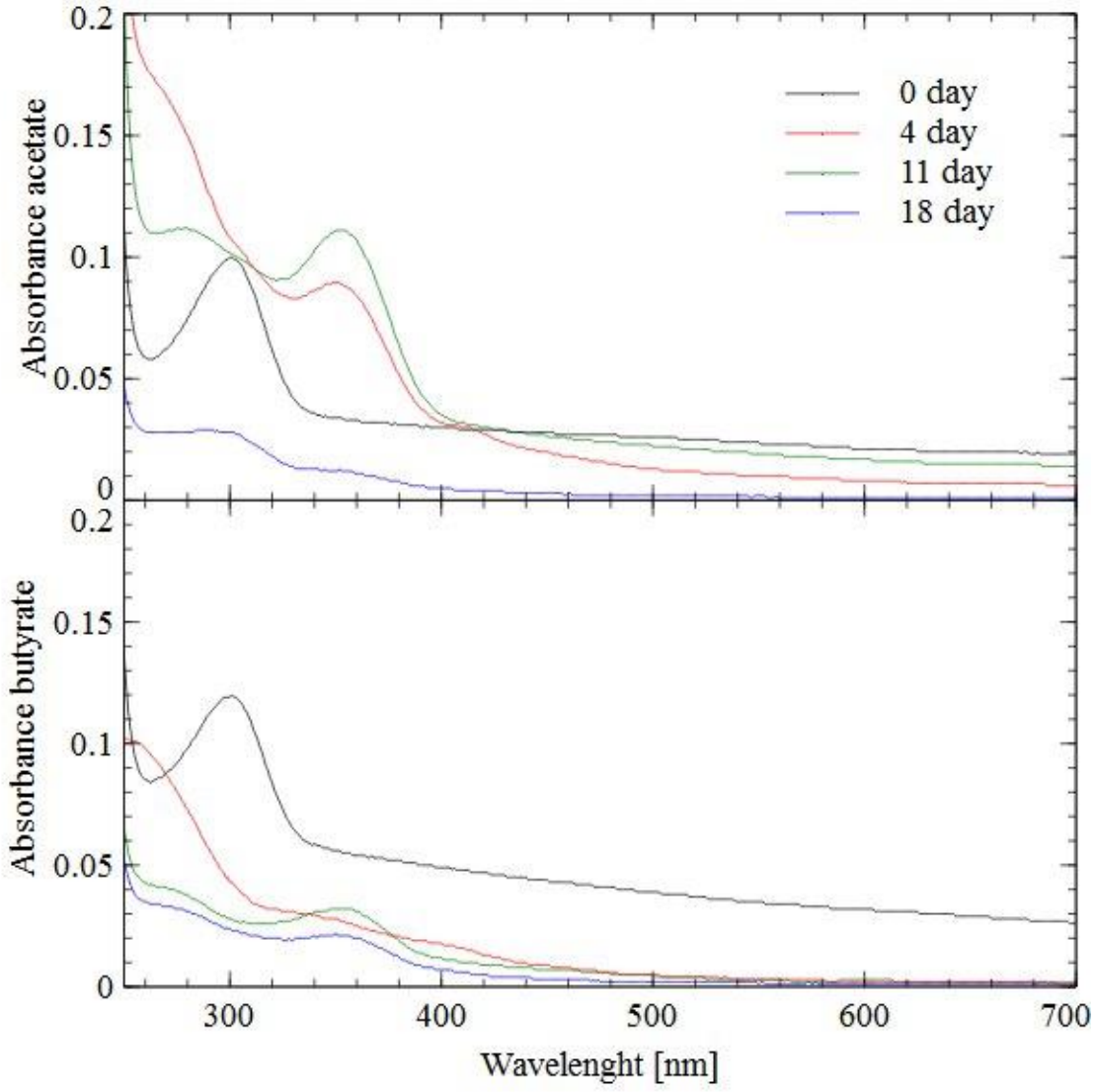

Figure 4.10: absorbance spectra of acetate and butyrate reactors of the second set

In acetate second reactor (Figure 4.10) two peaks appeared in correspondence of 250 and 350 nm denoting possible production of SMP species different from the respective

reactor in the first set. In butyrate reactor, the same peaks were registered as in the previous one but with a smaller intensity.

From the spectrum curves, it is possible to understand that compounds with different wavelength absorbance were produced in the reactors, even among those fed by the same carbon source, suggesting production of different SMP species. As previously mentioned, the only different parameters differentiating the first set from the second of experiments were the higher concentration of VFAs and the "age" of the microbial community. Therefore, the two mentioned parameters seem to influence the SMP species production.

Different intensity peaks were also registered, but there was no correlation with SMP concentration. The highest intensity was detected in the second acetate reactor, which showed the lowest SMP concentration, as shown in Figure 4.7. Therefore, this results suggested that the measured intensity cannot be correlated to the absolute SMP concentration and there are some SMPs that may not be detected by the spectrophotometer.

Slope ratio

The slope ratio S_R was demonstrated to be a good proxy for dissolved organic matter MW in different kinds of water. S_R are generally lower for the HMW fraction compared to the LMW region (Helms, Stubbins et al. 2008). In all the reactors, S_R values decreased with time. Therefore, the calculated values indicate a shift from LMW compounds to HMW along the course of the experiments. This result seems to be in accordance to the MW of SMP types. LMW compounds characterize UAPs, which are expected to be formed in substrate rich condition at the start of the experiment, while BAPs, which consist in HMW organic matter, are related to endogenous respiration at the end of the test when the substrate is consumed.

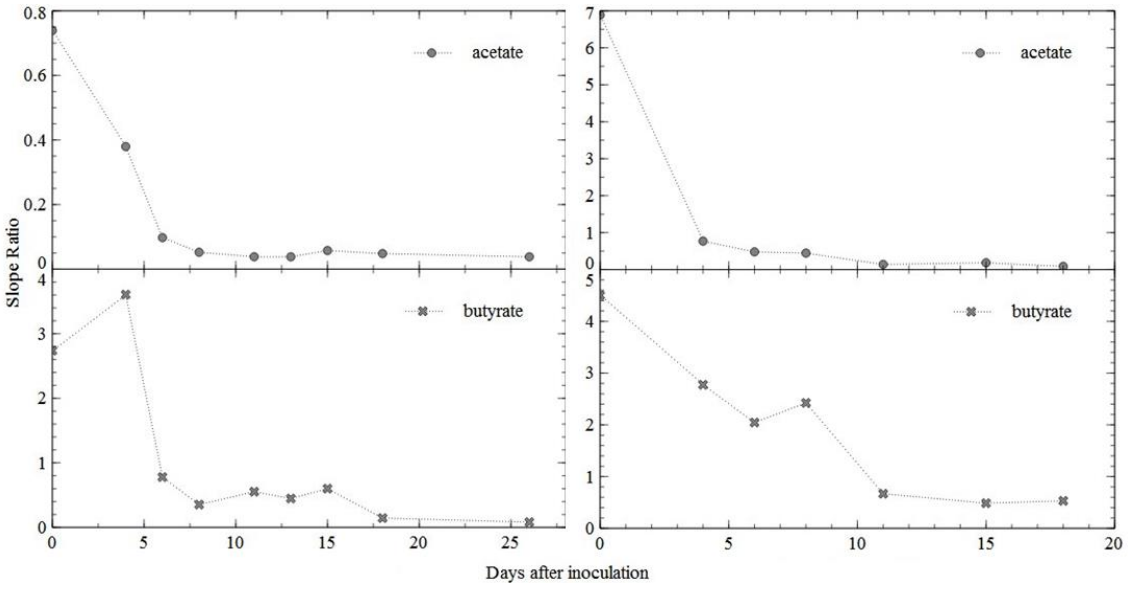

Figure 4.11: Slope ratio values for the first set of reactors (left) and second set (right)

SUVA

The Specific UV Absorbance (SUVA), determined at 254 nm, represents an index of aromaticity of the organic matter of the samples. In this wavelength range, the UV absorbance of DOC reflects the existence of unsaturated double bonds and π→π interaction like in aromatic compounds (Ates, Kitis et al. 2007).

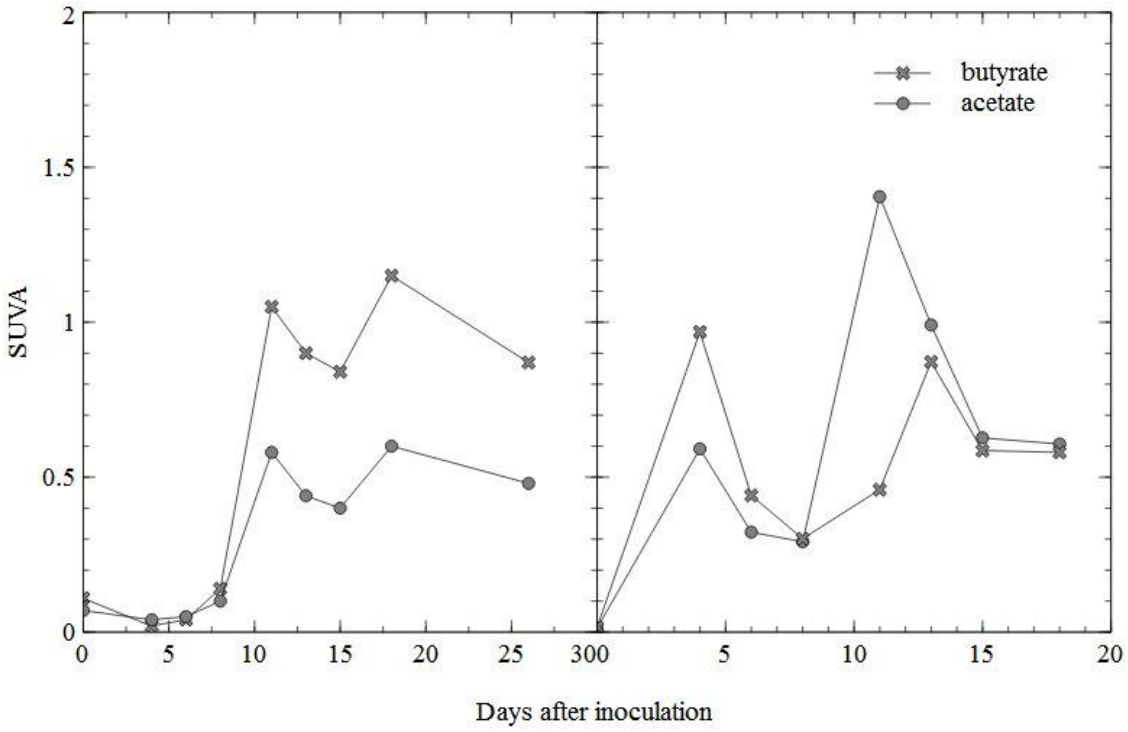

Figure 4.12: SUVA values distribution. On the left: first set. On the right: second set

In all the reactors, SUVA pattern exhibited two peaks followed by a declining curve as can be seen in Figure 4.12. This trend suggests a decomposition of biodegradable compounds and a remaining of refractory species (Jarusutthirak and Amy 2007). The increase of SUVA reflects that the fraction of larger, more aromatic and double bond rich compounds increased; therefore, the species produced at the peak of the curve are more hydrophobic and present low hydrogen to carbon ratio (Shin and Kang 2003). In general, the SUVA values measured at the end of the experiments are quite low <1.5 L/ mgCm. Natural waters with SUVA values <2-3 L/mgCm comprise mostly hydrophilic, non-humic and LMW compounds (Ates, Kitis et al. 2007).

4.1.7 MW distribution of organic matter

Figure 4.13, Figure 4.14, Figure 4.15, Figure 4.16 show the results of the HPSEC resulting from the transformation of VFAs in the activated sludge reactors. The molecular distribution of organic matter was determined using an online UV detection. This method has a limitation in the detection of low UV-absorptivity components, e.g. polysaccharides (Jarusutthirak and Amy 2007). All organic compounds could be recognized with an online TOC detector (Her, Amy et al. 2002), which is not present in

the Environmental Chemistry laboratory. Therefore, the results indicated just the UV sensitive fraction of SMP.

The MW distribution varied widely from very low (<100 Da) to high (>20 kDa). Several peaks were identified corresponding to MW of 194 Da, 1010 Da and 4040 Da. Organic matter with MW >200 kDa was detected towards the end of the experiments. In every reactors, very high intensity was registered at the beginning of the tests for compounds with MW <100 Da, probably justified by the fact that acetate and butyrate molar mass are in this range, respectively 59 Da and 87 Da. As previously mentioned, UAPs are mostly distributed in the LMW region (<100 Da), while BAPs are mostly composed of large molecules (>10 kDa).

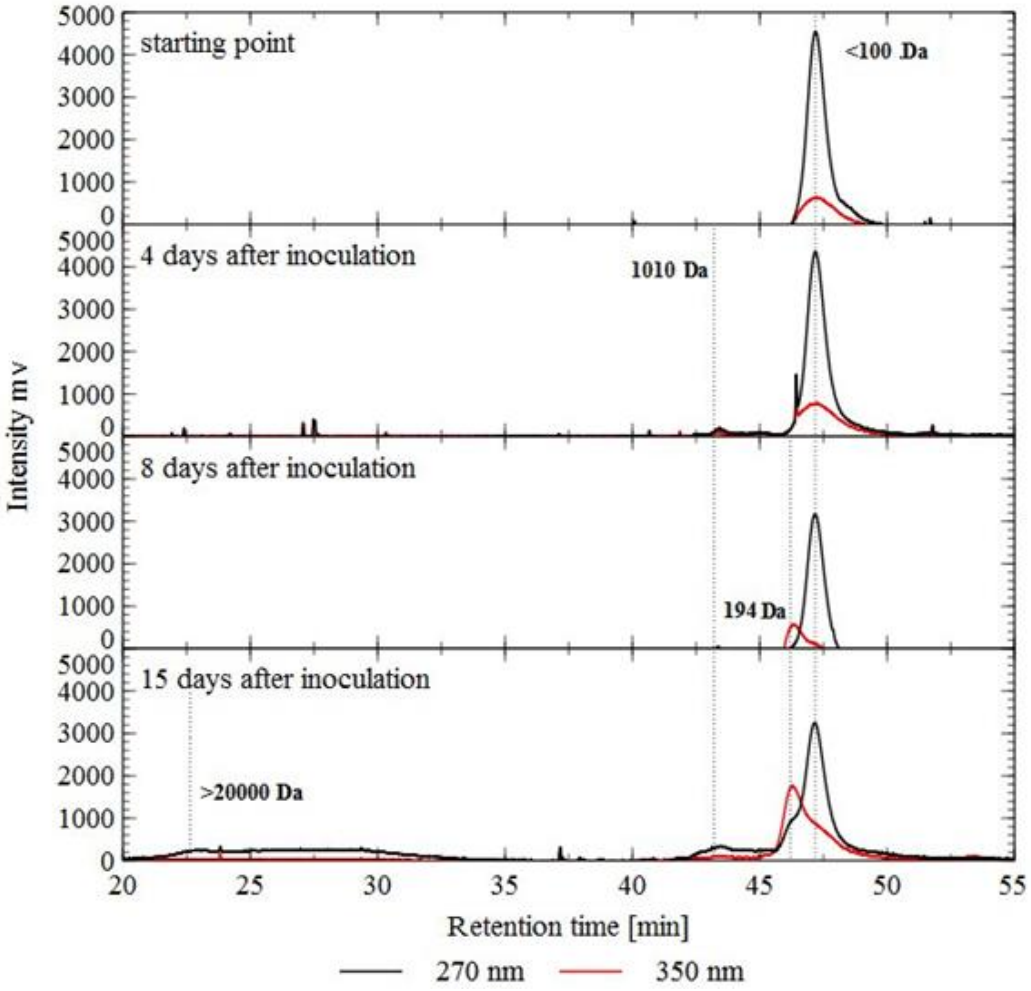

Figure 4.13: HPSEC results of the first set of acetate reactor

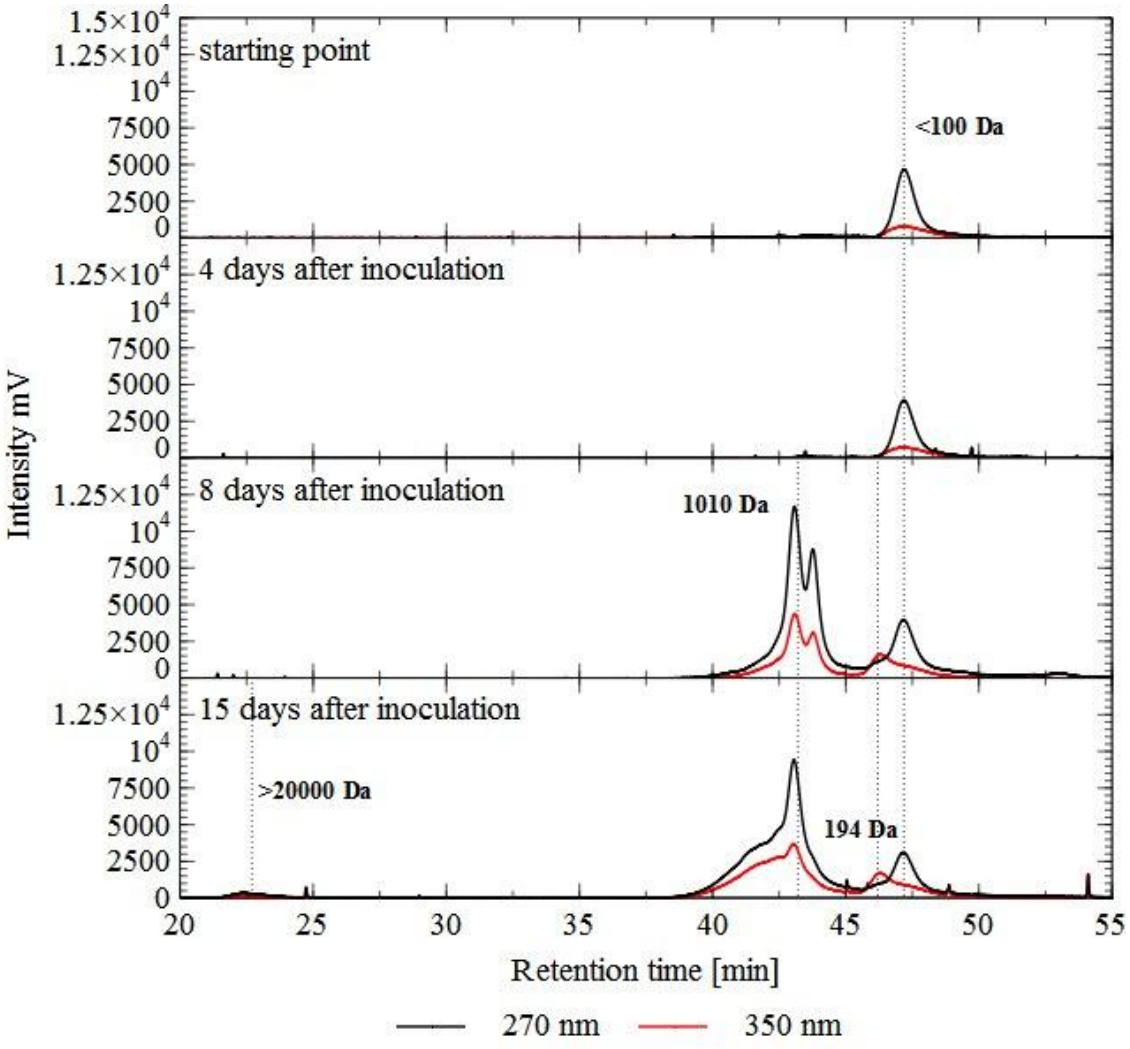

Figure 4.14: HPSEC results of the first set of butyrate reactor

Regarding the first set of experiments (Figure 4.13 and Figure 4.14), the chromatograms of acetate and butyrate reactors display the presence of LMW compounds, which intensity slightly decreased along the course of the test, probably reflecting the degradation of the volatile fatty acids. Even after acetate and butyrate complete depletion (day 11th), compounds with similar molecular weight were detected. This result suggest: (i) the presence of refractory UAPs and/or (ii) the formation of LMW compounds form by the degradation of bigger molecules, i.e. BAPs. In the butyrate reactor, eight days after the inoculation intermediates with higher MW (1010 Da) were formed and slightly degraded by the 15th day. The same MW compounds were detected in acetate reactor, but in a later stage of the inoculation and with lower intensity. HMW organic matter (>20 kDa) was detected in the samples collected 15 days after the inoculation, when the endogenous phase in those reactors had already started, as can be seen in the OD curves (Figure 4.1), suggesting that those compounds were BAPs. The intensities of the compounds measured at 350 nm were generally lower than the ones analyzed at 270 nm. On the other hand, the wavelength at 350 nm allowed detecting the

presence of compounds with a molar mass of 194 Da, which would have stayed unobserved with the test run at 270 nm.

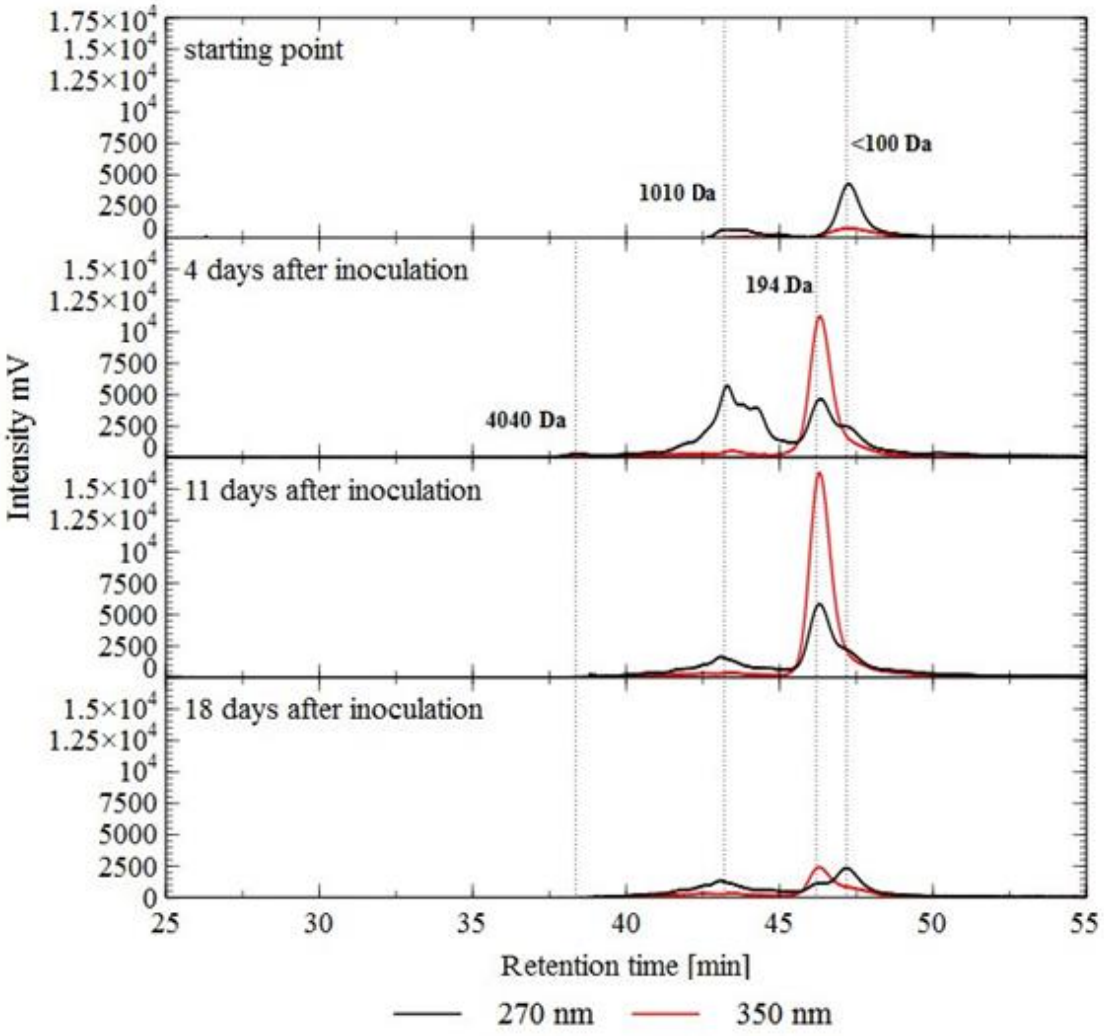

Figure 4.15: HPSEC results of the second set of acetate reactor

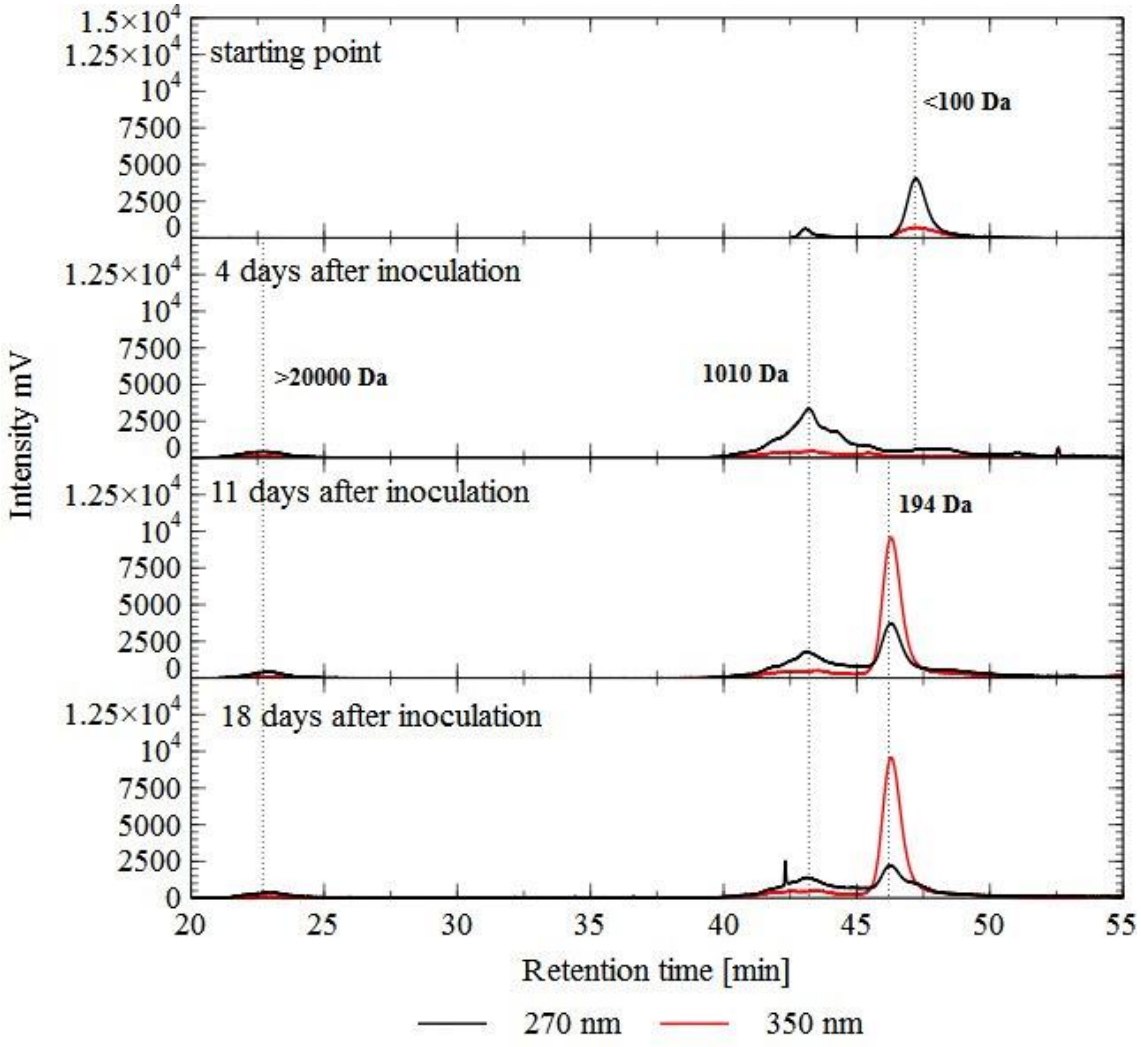

Figure 4.16: HPSEC results of the second set of butyrate reactor

Regarding the second set of experiments (Figure 4.15 and Figure 4.16), higher intensities were measured at 350 nm for compounds with a MW of 194 Da. The concentration of these species was high enough to be detected also at 270 nm. The LMW organic matter detected at the beginning of the test was almost completely degraded by the fourth day in both reactors. No HMW compounds seemed to be detected in acetate reactor, but this result does not exclude the presence of BAPs. There may be undetected SMPs in the samples that are not UV sensitive or have low absorption at 270 and 350 nm. In butyrate reactor, BAPs were observed since the fourth day, when low level of butyrate was measured. Therefore, starvation and biomass decay appeared to be the major factors responsible for those BAPs production.

Overall, the high molecular fraction of SMPs appear to increase along the inoculation time. A bimodal distribution was expected to be observed, with UAPs dominating at the beginning of the experiments during substrate rich conditions and BAPs prevailing towards the end for the period of the endogenous respiration. The intensities registered

in HMW region were low relatively compared to the LMW species, suggesting that most of BAP compounds remained undetected. Another explanation for the predominance of LMW compounds could be that HMW species were degraded or hydrolyzed to LMW, as suggested by (Shin and Kang 2003)

Comparison with spectroscopic methods

From the comparison of the results obtained with the spectroscopic method together with the HPSEC values, it was possible to conclude that the slope ratio S_R values were in accordance with the MW distribution, indicating a shift from LMW to HMW compounds.

Regarding the spectrum curves, from the observation of the intensity peaks registered for the same sampling days, it is possible to conclude that compounds with a MW of 194 Da had high absorbance at 350 nm, while compounds with a MW of 1010 Da showed a peak of absorbance at 260 nm. The peaks occurring at different retention times indicated the production of diverse species, supporting the absorbance spectrum results.

4.2 ISOLATION OF BACTERIOPHAGES

Isolation of Bacteriophages was attempted with bacterial isolates from the batch reactors. The isolation of bacteria was ensured by streaking the plate two times. A total amount of eight isolates was tested, four growing on acetate, two on butyrate, one on propionate and one on the general media. Figure 4.17 shows the microscopy image from isolated colonies grown on general media. The microscopy observation was not exhaustive with regard to establish the purity of isolates, since it could be one type of bacteria or different bacteria (bacilli) with the same shape.

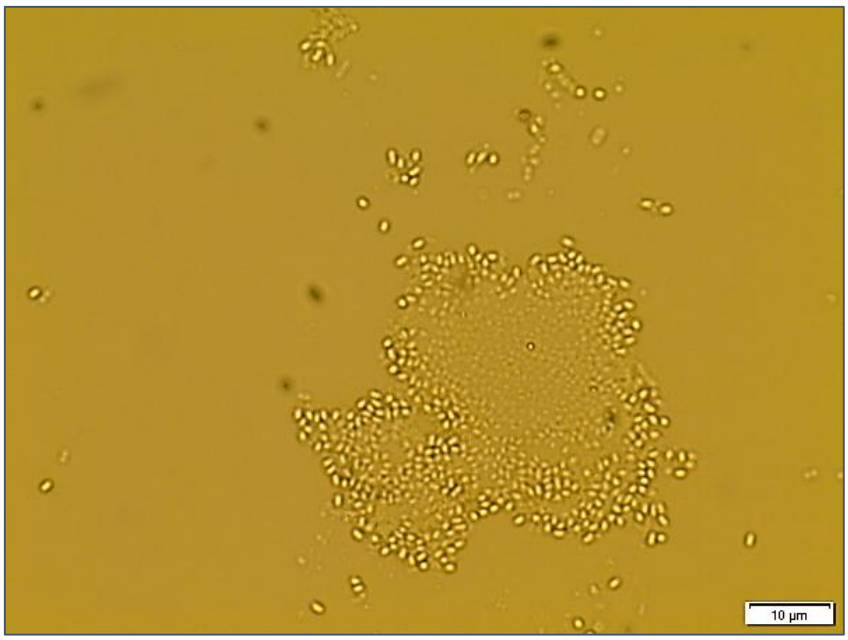

Figure 4.17: isolated bacterial population from the general media. Scale 10 µm

None of the plates showed plaque formation. This result may be justified by different explanation that are listed below.

i. The result may suggest that none of the isolates were host for the phages present in the activated sludge reactor. Since phage abundance is dependent on the concentration of its specific host, the isolated colonies need to be dominant in the investigated reactor in order to observe plaque formation (Shapiro and Kushmaro 2011). It is unlikely that the isolates from the batch reactors were representative of the dominants in the activated sludge basin, even though the inocula was taken from the same environment.
ii. Since the formation of the plaques depend on the conditions used, the failure in plaques observation may be due not to the lack of infection, but the impossibility to observe the lysis due to the experimental conditions (Hantula, Kurki et al. 1991), such as the culture media (Khan, Satoh et al. 2002). It is likely that some plates formed plaques so tiny to be impossible to detect with the naked eye.
iii. The isolation of the bacteria by the streaking method was assumed to be effective. Since no method was used to check the purity of isolates, such as gram staining, the bacterial culture may contain more than one strain. Hence, even if infection occurred on one isolate, plaque formation may have been impossible to observe because the area of cell lysis represent a nutrient rich zones for other bacteria to grow.
iv. Even though plaque formation was not detected, infection may have occurred in a form of lysogenic type, forming a dormant prophage (Khan, Satoh et al. 2002).

In some of the plates, the soft agar was dyed with methylene blue with the purpose of enhancing plaque observation. Even though the plaques were not observed, the dye enhanced the visualization of morphologies that could be interpreted as bacterial colonies. Figure 4.18 shows the possible colonies of bacterial isolates growing on acetate, propionate and butyrate respectively from left.

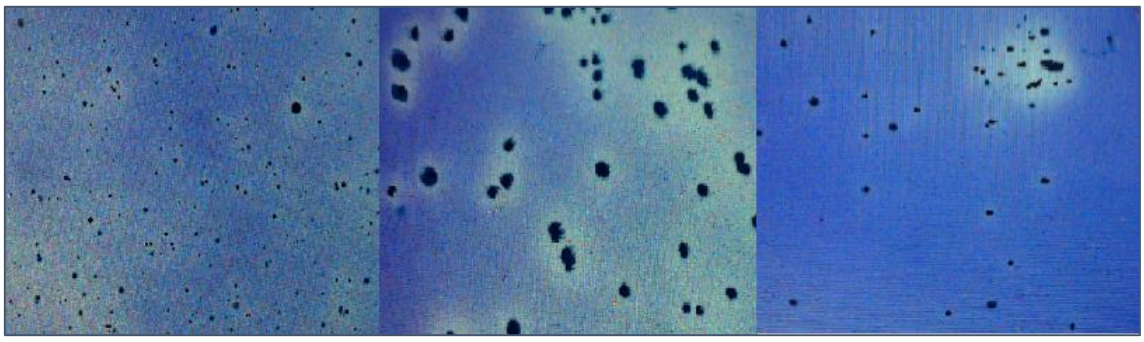

Figure 4.18: images of the plate for Bacteriophages isolation dyed with methylene blue

Due to the failure in observing plaque formation, it was not possible to performe any culture count over the plates. On the other hand, plaque assay cannot be considered a reliable method for giving an approximate number of phages in a diverse community

such the one found in activated sludge reactor, unless every host bacterium is isolated with an absolute specificity for a phage of the viruses community (Ewert and Paynter 1980). These requirements are impossible to meet, since just 1-20% of the bacteria population in the environment can be culturable (Andreottola, Baldassarre et al. 2002). Therefore, high proportion of viral diversity in activated sludge cannot be observed by this cultavion method (Wu and Liu 2009).

4.3 ENUMERATION OF BACTERIOPHAGES

The concentrations of bacteriophages at different stages of Rya wastewater treatment plant are listed in Table 4.4.

Table 4.4: Virus-like particle enumeration (Izon Science)

Stage of the plant	Mean diameter (nm)	Mode diameter (nm)	Concentration (VLP/mL)
Influent IN	91	90	$5.7 \cdot 10^{10}$
Activated sludge reactor AS	95	92	$2.1 \cdot 10^{10}$
Effluent EF	122	95	$2.3 \cdot 10^{9}$

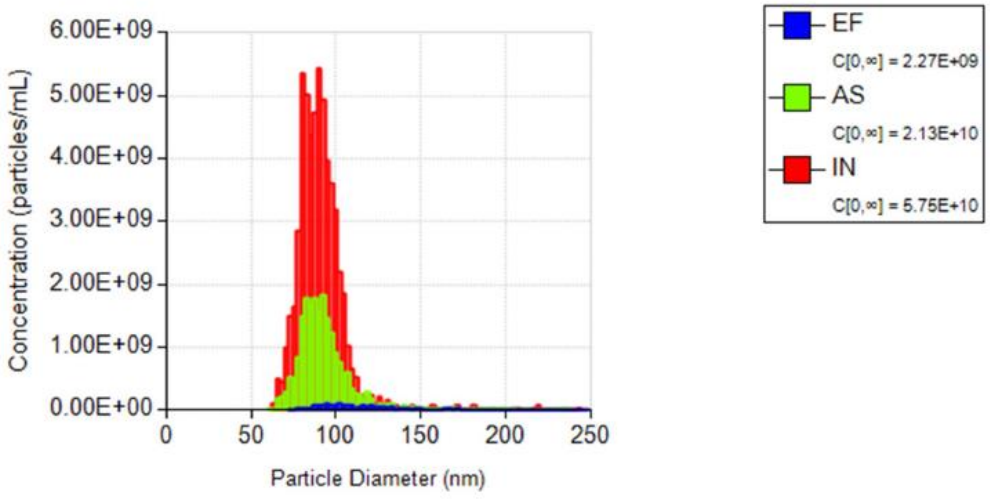

Figure 4.19: size histogram provided by Izon Science

The results reported a total viral count of 10^9 to 10^{10} virus like particles. Maximum concentration in the influent wastewater ($5.7 \cdot 10^{10}$ VLP/mL) was significantly higher than concentration in the effluent ($2.3 \cdot 10^9$ VLP/mL). The activated sludge reactor count

($2.1 \cdot 10^{10}$ VLP/mL) was slightly lower than the influent sewage. Hence, the measured values demonstrated a decreasing concentration of bacteriophages along the plant. The removal of viruses in the activated sludge processes occurs by adsorption to the flocs (Yasunori, Katsunori et al. 2002), therefore, high phage penetration to the sludge flocs may justify the relevant gap among the influent and the effluent. Bacteria, protozoa and metazoan may contribute also to the loss by ingesting the viral particles (Kim and Unno 1996). An opposite trend was obtained in the study of Ewert and Paynter (1980), in which the total concentration of phages in an activated sludge treatment plant was performed with direct electron microscopic counts. Their investigation showed an increase in number from the influent to the effluent, from $2.2 \cdot 10^{7}$ to $8.4 \cdot 10^{7}$ mL^{-1}, suggesting a net production of phages within the reactor (Ewert and Paynter 1980). The number measured is significantly lower than the results obtained; on the other hand it was argued that the method used in their investigation, transmission electron microscopy (TEM), may underestimate the count (Wu and Liu 2009). Wu and Liu applied epifluorescence microscopy and estimated the viral counts from $0.28 \cdot 10^{9}$ to $27.04 \cdot 10^{9}$ mL^{-1}. In their study, TEM was also applied to estimate the morphology of the viruses and they found that most of the viruses diameters ranges from 40 to 250 nm, similarly to the results obtained for Rya WWTP. Epifluorescence microscopy was used in another recent viral abundance study in Japan and the concentration was found to be between 10^{-7} to 10^{-9} (Otawa, Lee et al. 2007). The viral abundance in Rya WWTP along the water stream was higher than the study reported above. The explanation may be due to the different methods used. A comparison of the techniques would be useful to determine if Tunable Resistive Pulse Sensing (TRPS) overestimates the numbers of VLP. TEM has also the advantage to allow the characterization of virus morphology and diversity, which would help to differentiate the viruses in the activated sludge reactor from the influent and understand whether the viruses in the activated sludge are indigenous or are coming from the sewage inflow.

From phage enumeration, it was possible to estimate the contribution of VLP to the total dissolved organic carbon in the wastewater, assuming that 50% of the mass of the particle was carbon. Different DOC results were obtained considering diverse MWs. In the first case, the MWs were calculated through a correlation between size and mass of a protein molecule, assuming the following relationship (Erickson 2009):

$$\mathrm{MW(Da)} = \left(\frac{\mathrm{diameter(nm)}}{2 \times 0.066}\right)^{1/0.333}$$

Table 4.5: Molecular weight calculation from Erickson's correlation

	Particle mean diameter (nm)	Particle concentration (particles/ml)	MW from Erickson formula (Da)	MW from Erickson formula (g)
Influent IN	91	$5.7 \cdot 10^{10}$	$3.34 \cdot 10^{8}$	$5.54 \cdot 10^{-16}$
Activated Sludge AS	95	$2.1 \cdot 10^{10}$	$3.80 \cdot 10^{8}$	$6.31 \cdot 10^{-16}$
Effluent EF	122	$2.3 \cdot 10^{9}$	$8.06 \cdot 10^{8}$	$1.33 \cdot 10^{-15}$

In the other cases, the average mass of bacteriophages infecting E.coli cells named T2 and T5 was used, respectively $3.5 \cdot 10^{-16}$ and $1.9 \cdot 10^{-16}$ g. The calculated DOC results are shown in Table 4.6.

Table 4.6: contribution of the VLP to the wastewater DOC

	DOC using Erickson's correlation(mg/L)	DOC using T2 average mass (mg/L)	DOC using T5 average mass (mg/L)
Influent IN	15.81	19.95	10.83
Activated Sludge AS	6.63	7.35	3.99
Effluent EF	1.54	0.81	0.44

DOC concentrations were measured in Rya wastewater effluent in the same period of sampling for bacteriophages enumeration (January-February). The average value resulted in a DOC equal to 7.6 mg/L. Hence, the VLP may contribute to the effluent DOC in a percentage varying from 5.8 to 20.2%. The results show that phages could be a significant contributor to the DOC in the effluent from the plant, when present in high concentration (10^{10}). Since it was found that temperature affects bacterial growth and phage production inversely (Warner, Barker et al. 2014), the contribution of phages could be lower during warmer months than in wintertime, when the enumeration was performed.

5 CONCLUSION

Based on the growth media, different population of aerobic heterotrophic bacteria were enriched in the reactors, characterized by different growth curves and kinetic coefficients. The peaks in cell density reflected the free energy values obtained from the carbon source, except for propionate fed reactor, which showed the highest biomass yield and cell density. The free energy values seem also to be correlated to SMP production. The higher was $\Delta G°$ obtained from the compound the higher was the SMP maximum concentration observed.

SMPs were produced since the start of the experiment in high concentration, with the highest accumulations occurred in the growth phases of the communities in abundant substrate condition, suggesting that their formation is a relatively fast process. Even though in the second set of reactors the VFA concentration was three times higher, the detected SMP concentration was lower, proposing that the longer a community is acclimatized to a carbon source the lower is SMP production.

The calculated spectrophotometric parameters gave information about the aromatic nature of the DOC and the change in MW along the time. SUVA results indicated that the produced SMP comprised mostly hydrophilic, non-humic and LMW compounds. The S_R values decreased with time, indicating a shift from LMW compounds to HMW along the course of the experiments. The diverse shape and intensity of the spectrum curves indicated that compounds with different wavelength absorbance were produced in the reactors, even among those fed by the same carbon source, suggesting production of different SMP species. HPSEC results confirmed the findings from S_R values and absorbance spectra. The MW distribution varied widely from very low (<100 Da) to high (>20 kDa) with a transition from LMW to HMW. Several peaks were identified corresponding to MW of 194 Da, 1010 Da and 4040 Da, indicating the formation of different compounds. It is possible to conclude that the spectrophotometric method is a practical and reproducible technique giving a first and fast characterization of SMP, using a small volume of sample that does not required sophisticated sample pretreatment and analytical equipment. No information about SMP concentration or identification of the compounds are provided since the method is limited to detection of light-absorbing compounds.

Comparing the results obtained in each reactor, it is possible to conclude that the carbon source, its concentration and the acclimatization of the microbial community may influence SMP productivity in terms of concentration and composition.

The bacteriophages isolation failed due to lack of plaque formation, which can be justified by the following explanations: absence of host for phages, not suitable experimental conditions, contamination in the bacterial isolates, and occurrence of lysogenic infection.

High dynamic of virus abundance characterizes the water stream of Rya WWTP, with higher abundance in the influent and activated sludge reactor compared to the effluent. When present in high concentration (10^{10} mL^{-1}), VLP may constitute a significant

fraction of the total DOC in the wastewater effluent. The viral count was significantly higher than the investigation performed in other WWTPs. Therefore, it remains to determine whether Tunable Resistive Pulse Sensing (TRPS) overestimates the numbers of VLP and whether the temperature of wastewater influences the count of bacteriophages.

5.1 RECOMMENDATIONS

The following is a list of recommendations if future experiments are carried out with SMP investigation in batch reactors or bacteriophages isolation and enumeration from activated sludge processes.

i. Since the production of SMPs seems to be a fast process, multiple samples should be taken in the first hours just after the biomass gets in contact with the substrate.
ii. In order to detect all the organic compounds and not just the UV sensitive fraction, size distribution analysis should be carry out using ultrafiltration or SEC with online TOC detector.
iii. If performing UV analysis over the spectrum, it is recommendable to use a liquid nutrient media without NO_3^-, since its absorption is elevated and disturb the absorption of the other compounds.
iv. It would be useful to assess the biodegradability of the SMP parallel to the quantity produced by different reactors, since lower production may also involve the production of more refractory compounds.
v. In order to be successful, bacteriophages isolation with the method of the plaque assay should be attempted just on dominant bacteria of the same phage environment.
vi. Different media culture should be tested for bacteria and bacteriophages isolation, since this condition may inhibit the plaque formation. A recommended culture media from the literature contains sodium acetate 5g/L, tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, $MgSO_4 \cdot 7H_2O$ 0.2g/L, $MnSO_4 \cdot 7H_2O$ 0.05 g/L, and agar 15 g/L (hard agar) or 4 g/L (soft agar) (Khan, Satoh et al. 2002). In this study, 15 bacterial isolates were obtained from activated sludge process and nine of them supported plaque formation.

APPENDIX

Kinetic and Stoichiometric coefficient calculation

Table A 2 contain the experimental data used for the coefficient calculations.

Table A 1: VFA and MLVSS concentration from the first set of reactors

	Acetate		Propionate		Butyrate	
Days	VFA [mg VFA-C/L]	MLVSS [mg VSS/L]	VFA [mg VFA-C/L]	MLVSS [mg VSS/L]	VFA [mg VFA-C/L]	MLVSS [mg VSS/L]
0	203.21	14.00	213.30	14.00	165.22	14.00
4	171.00	20.00	195.46	15.00	157.12	23.50
8	67.97	26.00	129.10	30.00	90.77	47.00
11	0.00	44.00	0.00	57.50	12.27	33.00
13	0.00	38.50	0.00	64.75	13.98	46.75
15	0.00	38.50	0.00	53.00	11.58	36.50
18	0.00	34.25	0.00	47.25	12.54	31.75
20	0.00	32.00	0.00	44.75	0.00	37.00
26	0.00	21.75	0.00	29.00	9.02	30.25

Table A 2: VFA and MLVSS concentration from the second set of reactors

	Acetate		Butyrate	
Days after inoculation	VFA [mg VFA-C/L]	MLVSS [mg VSS/L]	VFA [mg VFA-C/L]	MLVSS [mg VSS/L]
0	535.56	14.50	587.92	17.25
4	6.25	96.25	5.58	169.00
6	0.00	78.00	2.72	120.00
8	0.00	79.60	22.15	111.20
11	0.00	67.75	68.42	79.75
13	0.00	51.25	59.19	76.50
15	0.00	55.75	5.83	49.25
18	0.00	41.00	6.32	56.00

The figures below show the biomass development and substrate utilization graphs with experimental data and fitted lines using the calculated coefficients.

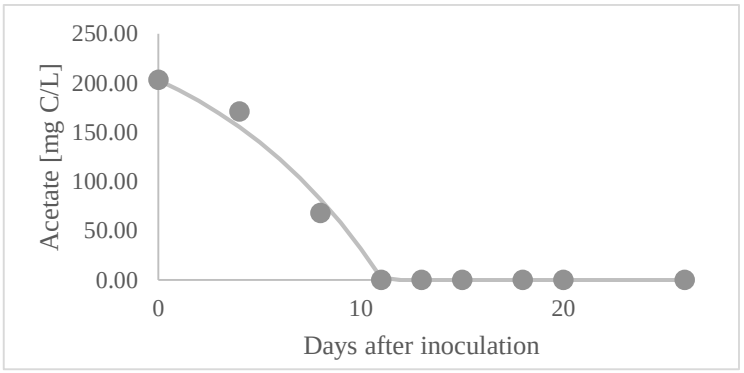

Figure A 1: measured and modelled values for acetate consumption in the first set of experiments

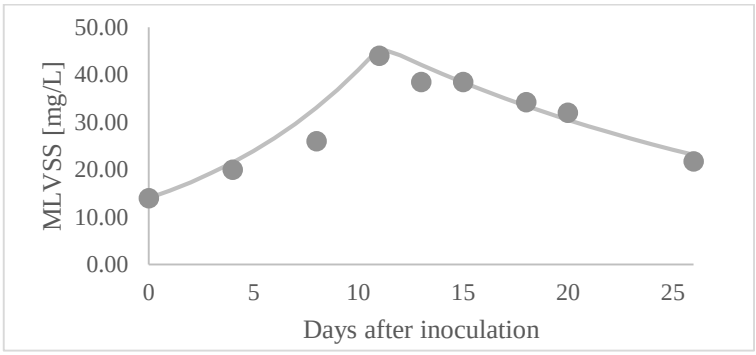

Figure A 2: measured and modelled values for biomass development for acetate reactor in the first set of experiments

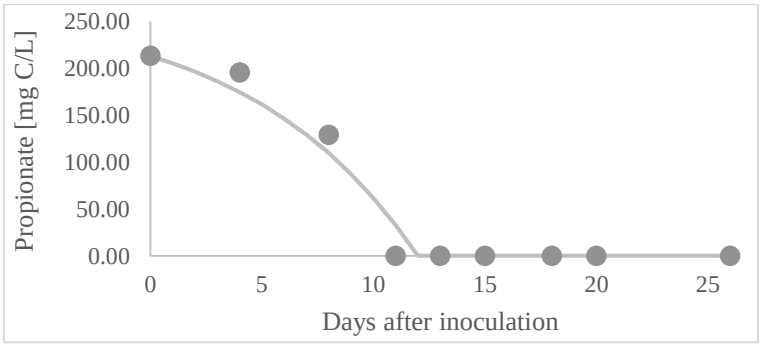

Figure A 3: measured and modelled values for propionate consumption in the first set of experiments

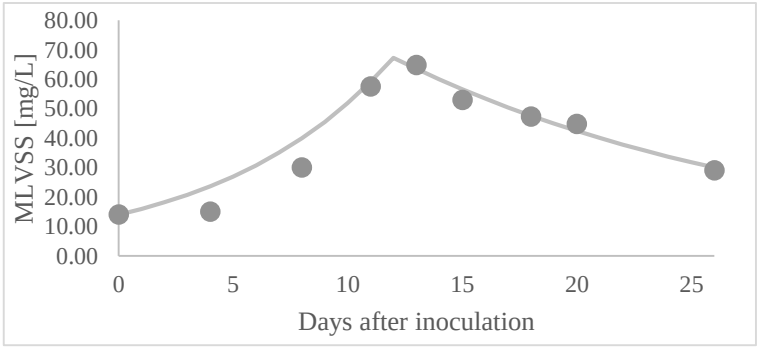

Figure A 4: measured and modelled values for biomass development for propionate reactor in the first set of experiments

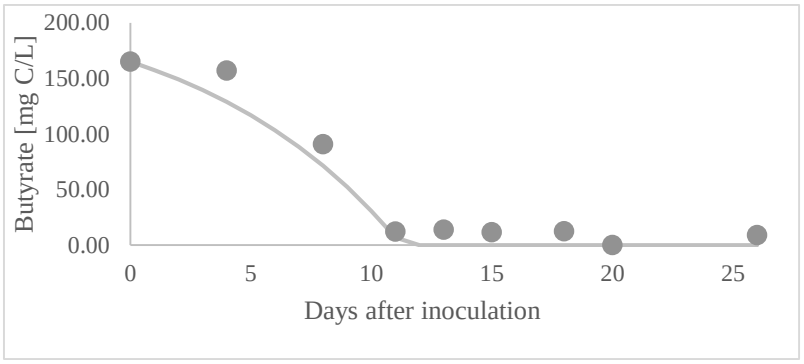

Figure A 5: measured and modelled values for butyrate consumption in the first set of experiments

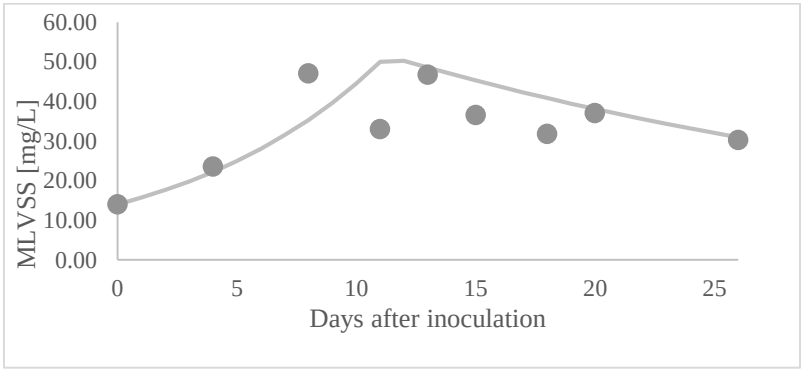

Figure A 6: measured and modelled values for biomass development for butyrate reactor in the first set of experiments

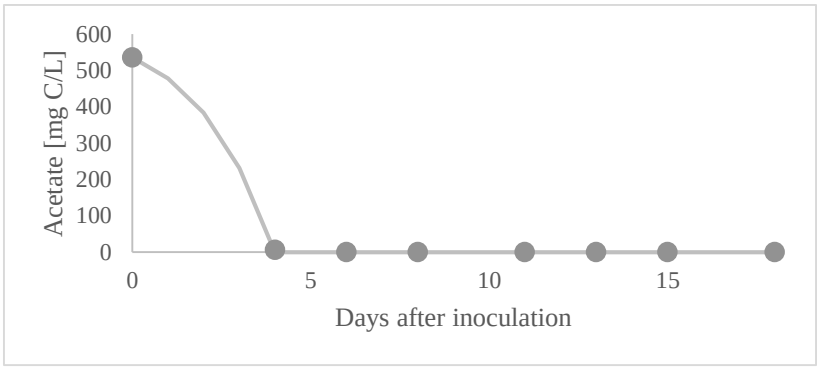

Figure A 7: measured and modelled values for acetate consumption in the second set of experiments

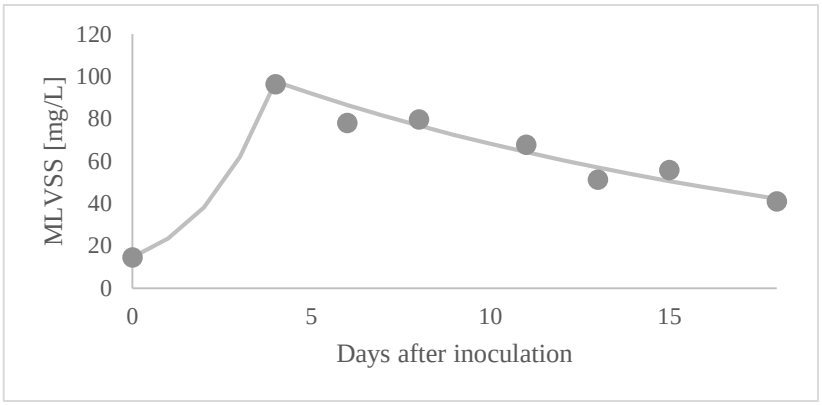

Figure A 8: measured and modelled values for biomass development for acetate reactor in the second set of experiments

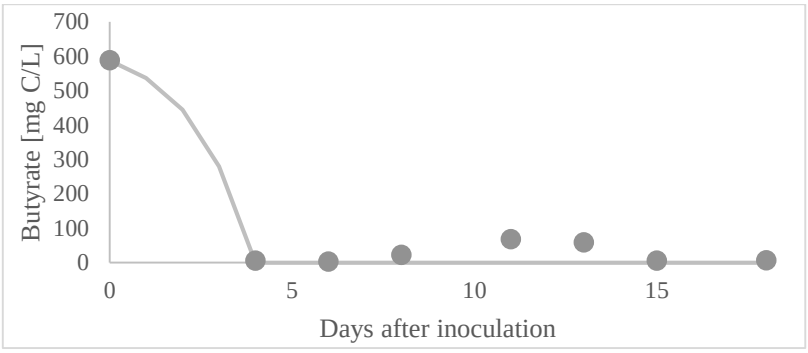

Figure A 9: measured and modelled values for butyrate consumption in the second set of experiments

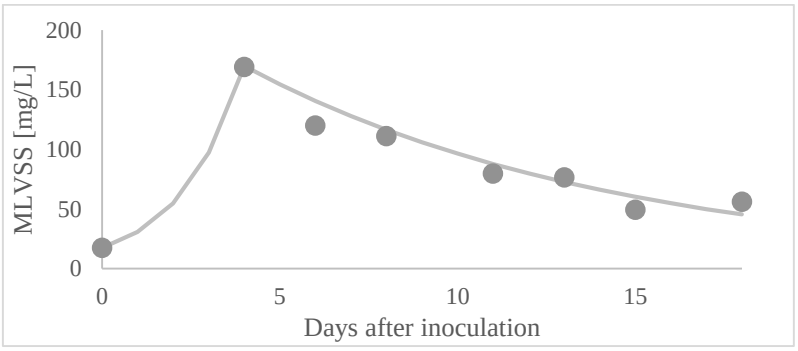

Figure A 10: measured and modelled values for biomass development for butyrate reactor in the second set of experiments

Spectrum of the nutrient media

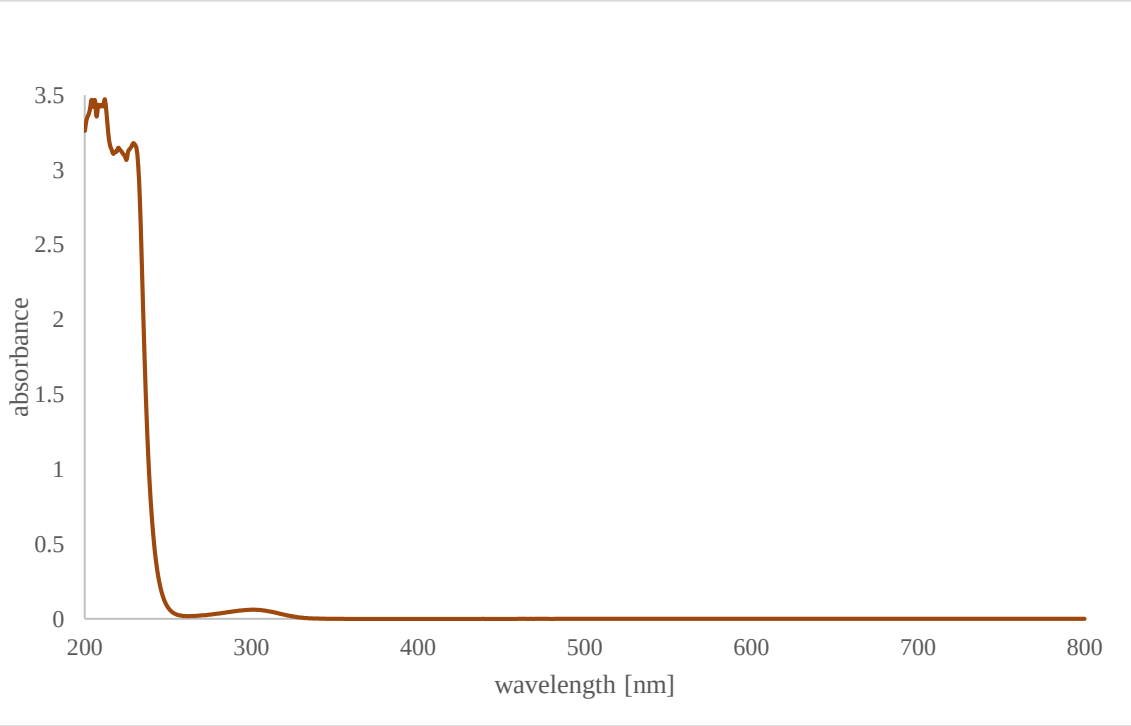

Figure A 11: absorbance spectrum of the media

Microscopy investigation

Figure A 12 shows the images observed with the light microscope of morphologies that could be interpreted as bacterial colonies in the plates dyed with methylene blue.

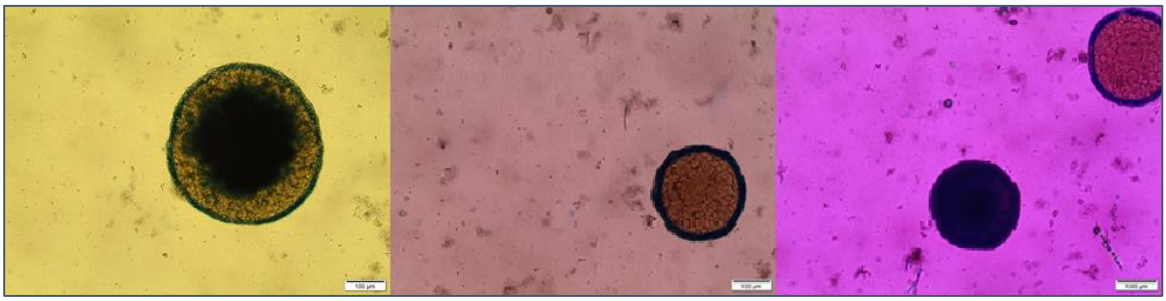

Figure A 12: image of possible bacterial colonies appeared on the plates dyed with methylene blue. Scale: 100 µm

In Figure A 13, it is possible to observe a flock of a sample taken from acetate fed reactor at the end of the experiments. At the moment of the sampling, the carbon source had already been depleted since many days. In this starvation stage, no filamentous bacteria were observed in the samples.

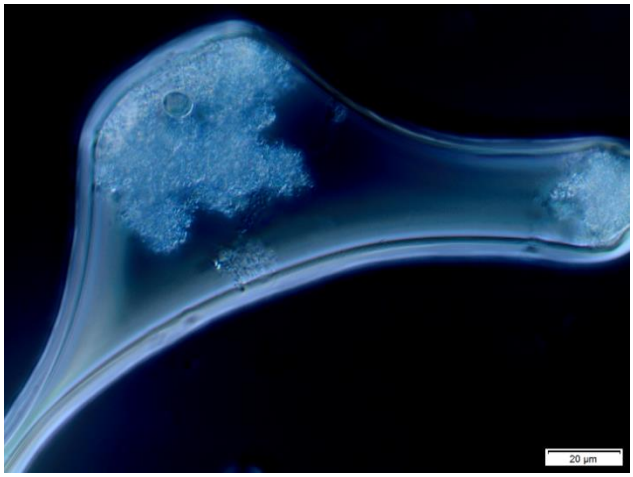

Figure A 13: image of a flock in acetate fed reactor. Scale: 20 µm

Figure A 14 shows a rotifer feeding on the flock of butyrate fed reactor. Rotifers thrive in aerobic condition and are indicator species for stable activated sludge systems.

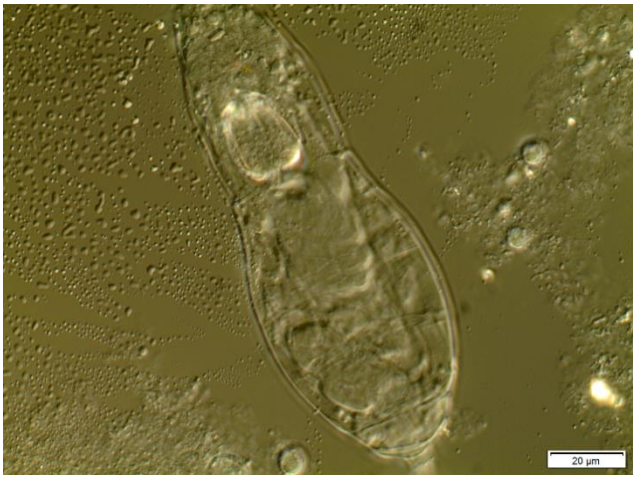

Figure A 14: image of a rotifer feeding on the flock of butyrate fed reactor. Scale: 20 µm

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